The Impact of Sugar Derived Metabolites (AGEs) on Pubertal Mammary Gland Development

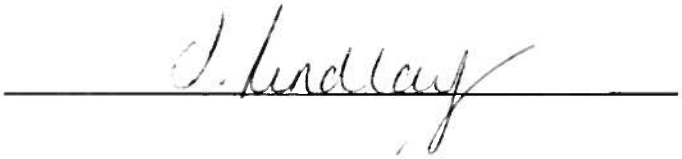
by **Bradley Krisanits**

A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Masters of Biomedical Sciences in the College of Graduate Studies.

Department of Pathology and Laboratory Medicine, 2017

Approved by:

(Chairman, David P. Turner) 

(Victoria J. Findlay) 

(Kristi L. Helke) 

(Elizabeth Yeh) 

(Robin Muise-Helmericks) 

Copyright Page

Acknowledgments

My desire for a career in biomedical research has always been a strong passion of mine. However, without the guidance and education of my mentor, Dr. David Turner I would not have been able to reach my fullest potential and to further my career. Thank you for welcoming me into your lab, pushing me to branch out and to think independently, ultimately pushing me to become a true scientist. I feel that with your guidance I am now truly prepared for the road that lies ahead on my path to a successful career in science.

I would also like to thank Dr. Victoria Findlay and the members of the lab for their support and making me feel at home from the start. Whether it be demonstration of techniques, or aiding in an experiment, to eating all the office candy. This research could not have been completed without the guidance of Dr. Findlay and all the members of the lab. In addition, thank you to the other members of my advisory committee, Dr. Elizabeth Yeh, Dr. Kristi Helke and Dr. Robin Muise-Helmericks, for taking the time out of your busy schedules to spend hours on meetings, assisting with my research and reading/critiquing my work. I know I will be a better research scientist because of both your guidance and support. Also, thank you to the department of Pathology and Laboratory Medicine at the Medical University of South Carolina for making this experience possible.

Lastly, I would like to acknowledge my friends and family for all their unconditional support throughout my relentless desire for a career in biomedical research. Thank you to my parents for sacrificing plenty for me to obtain a proper education and for being there during the stressful times and long phone calls to push me through, without you, none of this would have been possible. Thank you all.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADH – Atypical Ductal Hyperplasia

AGEs – Advanced Glycation End-products

AIF – Apoptosis Inducing Factor

AREG – Amphiregulin

BRCA1 – Breast Cancer 1

CAFs – Cancer-associated Fibroblasts

CCL5 – Chemokine Ligand 5

CSF-1 – Colony-Stimulating Factor 1

CSF-1R – Colony-Stimulating Factor 1 Receptor

CXCL1/2 – Chemokine Ligand 1 and 2

DAB – 3,3'-Diamino-benzidine

DCIS – Ductal Carcinoma In-situ

diH_20 – Deionized Water

DMBA – 7,12-dimethylbenz[a]anthracene

E_2 – Estrogen

ECM – Extracellular Matrix

EGF – Epidermal Growth Factor

EMT – Epithelial-Mesenchymal Transition

ER – Estrogen Receptor

EtOH – Ethanol

FGF – Fibroblast Growth Factor

GH – Growth Hormone

GM-CSF – Granulocyte Macrophage Colony Stimulating Factor 1

H&E – Hematoxylin and Eosin

HCl – Hydrochloric Acid

HGF – Human Growth Factor

Hiflα – Hypoxia-Inducible Factor 1-Alpha

ICAM-1 – Intracellular Adhesion Molecule 1

IDC – Invasive Ductal Carcinoma

IGF1 – Insulin Growth Factor 1

IHC – Immunohistochemistry

IL-1β – Interleukin 1 Beta

IL-4 – Interleukin 4

IL-6 – Interleukin 6

IL-8 – Interleukin 8

IL-10 – Interleukin 10

IL-13 – Interleukin 13

iNOS – Inducible Nitric Oxide Synthase

LN – Lymph Node

MAPK – Mitogen Activated Protein Kinases

MIN – Mammary Intraepithelial Neoplasia

MMP – Matrix Metalloproteinase

MMP-2 – Matrix Metalloproteinase 2

MSCs – Mesenchymal Stem Cells

n-3 PUFA – n-3 polyunsaturated fatty acids

n-6 PUFA – n-6 polyunsaturated fatty acids

NAD(P)H – Nicotinamide Adenine Dinucleotide Phosphate-oxidase

NF-κB – Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells

NH_4OH – Ammonium Hydroxide

PFA – paraformaldehyde

PI3K – Phosphatidylinositol-3-kinases

PRRs – Pattern-recognition Receptors

RAGE – Receptor for Advanced Glycation End Products

ROS – Reactive Oxygen Species

RT – Room Temperature

SDF-1 – Stromal Cell-Derived Factor 1

αSMA – Smooth Muscle Actin

STAT3 – Signal Transducer and Activator of Transcription 3

TAM – Tumor Associated Macrophage

TDLU1 – Terminal Ductal Lobular Unit 1

TEB – Terminal End Bud

TED – Terminal End Duct

TGF-β – Transforming Growth Factor-Beta

TNF-α – Tumor Necrosis Factor Alpha

VCAM1 – Vascular Cell Adhesion Protein 1

VEGF – Vascular Endothelial Growth Factor

Abstract

The mammary gland is one of few organs that continues to develop postnatally, through puberty, pregnancy, lactation and involution. Interestingly, these times of development are also considered windows of susceptibility for future development of breast cancer. Studies have shown that processes important in mammary gland development are often deregulated during breast cancer tumorigenesis.

Advanced glycation end products (AGEs) are highly reactive metabolites produced during normal metabolism. AGE content in the Western Diet has consistently increased over the years and accumulation of AGEs in the body leads to pro-inflammatory and pro-oxidant effects. Diet, including a diet high in fat has been shown to deregulate mammary development and lead to increased breast cancer risk. This study examines the impact of a diet high in AGEs on pubertal mammary gland development in mice. Mice were fed a control and high AGE diet from onset of puberty (3 weeks) to experimental endpoints of 7, 8 (pubertal) and 12 weeks (adulthood). At each experimental endpoint blood was collected for analysis of circulating AGEs and mammary gland tissue was collected for whole mounting, immunohistochemistry of AGE, RAGE, Ki67, αSMA, and F4/80 as well as morphology with hematoxylin and eosin (H&E). Mice fed the high AGE diet showed increase ductal branching, terminal end bud (TEB) number and size, as well as a delay of ductal extension. Immunohistochemistry techniques showed increased proliferation (Ki67) and disruption of myoepithelial layer (αSMA) in mice fed a high AGE diet. Histology revealed changes in morphology of gland structures, increased recruitment of stromal cells surrounding ductal/TEB structures, including macrophages and activated fibroblasts, as well as the formation of pre-neoplastic like lesions in adulthood with mice fed a high and low AGE diet.

In summary, we observed a significant disruption of normal pubertal mammary gland development in mice fed a high AGE diet when compared to mice fed a control diet, possibly leading to increased breast cancer risk in adulthood.

Introduction and Background

Advanced Glycation End-Products.

Advanced glycation end-products (AGEs) are reactive metabolites formed via normal metabolism through a reaction commonly known as the Maillard reaction (Figure 1). This reaction occurs in a series of three phases: the first being glycation, or the reversible non-enzymatic reaction causing the addition of reducing sugars to the amino groups on biological macromolecules, such as: proteins, lipids or nucleic acids. This leads to the formation of Schiff bases. The second step involves a series of reversible rearrangements of the Schiff bases to form early glycation precursors, or Amadori products. Accumulation of these early glycation precursors leads to further rearrangements irreversibly forming AGE reactive metabolites [1-5].

The Maillard reaction, while the main source of AGE formation in the body is not the only pathway leading to the formation of these reactive metabolites (Figure 1). Autoxidation of glucose and the peroxidation of lipids by increase oxidative stress lead to the formation of dicarbonyl derivatives, known as α-oxaldehydes. These derivatives can interact with monoacids to form AGEs. Glucose can also be converted to sorbitol by aldose reductase and then to fructose by sorbitol dehydrogenase, a reaction known as the polyol pathway. Fructose metabolites can then be converted to α-oxaldehydes, which again can interact with monoacids and form AGEs. Given the different pathways that AGEs may form through, it is no surprise that AGEs structurally are diverse (Figure 2) [1-5].

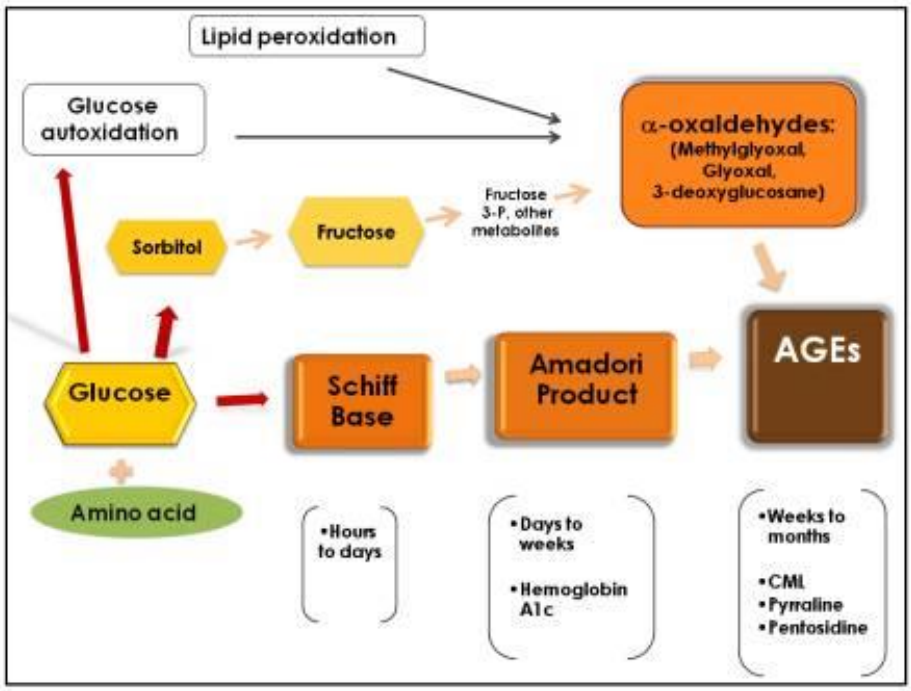


Figure 1. Mechanisms of AGE Formation. Endogenous formation of AGEs can occur through a variety of mechanisms. The Maillard reaction being the classic pathway, but AGEs can also be formed through glucose autooxidation, lipid peroxidation, and the polyol pathway. Taken from "Dietary Advanced Glycation End Products and Aging" by C. Luevano-Contreras and K. Chapman-Novakofski, 2010, Nutrients, 2(12):1247-1265.

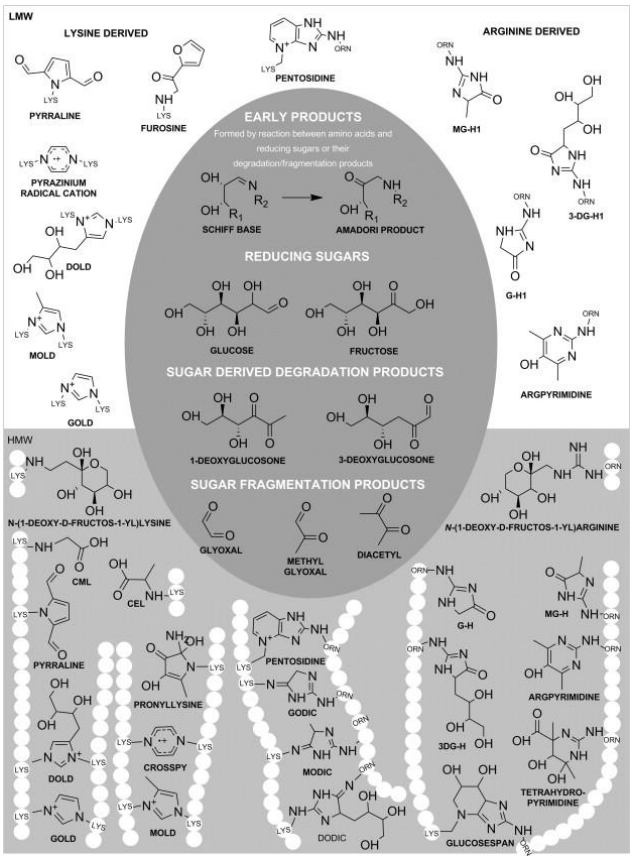


Figure 2. Variety in AGE Structure. Structures of various low molecular weight (LMW) and high molecular weight (HMW) AGEs derived from the amino acids lysine, arginine, or both showing variation of AGE formation on structure. Initial intermediate products of AGE formation are shown in the middle, with the structure of common reducing sugars with their degradation and fragmentation products. Taken from "Advanced glycation end-products in food and their effects on health." by M. Poulsen et al., 2013, Food and Chemical Toxicology 60: 10-37.

AGEs are naturally found in uncooked foods, but AGE content can be accelerated by cooking methods, such as grilling, broiling and searing, which has shown to induce the greatest AGE formation [1, 4-6]. Processed foods treated for preservation can also increase AGE content in sugar laden foods [6]. AGEs are also being added directly to highly processed foods to increase appearance and taste as we have developed overtime to enjoy the taste of AGEs [1, 4-6]. Cigarettes and processed

tobacco smoking are also an exogenous source of AGEs, as glycotoxins inhaled can be transported into the blood stream and thereby contribute to AGE formation [1-5].

The Western diet generally consists of a diet that is high in sugar, fat and processed foods, it has become more prevalent over the years and shown to contribute to the dramatic increase of obesity and type 2 diabetes [7]. These foods have also been shown to have a high AGE content. Along with an increase in western diet, sedentary lifestyle has also risen, further contributing to the accumulation of AGEs in the body as an active lifestyle was shown to decrease AGEs in the circulation [1-6, 8].

AGE Reduction. A main lifestyle intervention aimed at reducing AGE accumulation in the body is restriction of foods containing a high AGE content [2-6, 9]. Several such intervention studies in both animal and human subjects showed that intake of AGEs from diet contribute to tissue damage that can be monitored by dietary restriction. Animal models such as *C. elegans* and mice have been shown to have increased lifespan upon caloric restriction leading to decrease in AGE accumulation and oxidative stress [3, 4, 8, 9]. Exercise has also been shown to lead to a decrease in AGE accumulation in both animal and human models, something we have observed in our own clinical trials with breast and prostate cancer patients (unpublished observations), while some pharmacological interventions such as metformin and Orlistat have shown promising results in reduction of AGE formation in pre-clinical and clinical trials, it is unorthodox however to distribute such drugs to adolescents without knowing the side-effects [3-6, 9]. Antioxidants, such as melanoidins, catechins, proanthocyanidins and phenolic antioxidants have also shown a promising role in the reduction of AGE formation and oxidative stress, giving adolescents healthier options of AGE reduction [2-6, 9].

Receptor for Advanced Glycation End-Products, AGE Signaling and Cancer.

While AGEs can act independently of a receptor, leading to damage of protein structure and function, often seen in cross-linking of collagen in the extracellular matrix (ECM), they can also act as a ligand for the receptor of advanced glycation end-products (RAGE) [7, 9-13]. RAGE is a pattern recognition transmembrane receptor that is a part of the immunoglobulin superfamily (Figure 3) [7, 9-13]. While it is expressed at low levels in all tissues on multiple cell types, including macrophages and epithelial cells, it can be greatly increased in pathophysiological settings, such as diabetes, Alzheimer disease and cancer [7, 9-14]. Interestingly, RAGE signaling in macrophages led to increased proliferation, recruitment and maturation [7, 9-13].

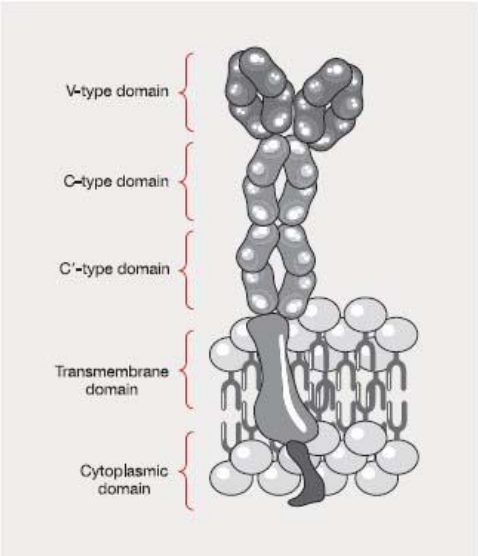


Figure 3. RAGE structure. Taken from "Receptor for Advanced Glycation End Products and Atherosclerosis: from Basic Mechanisms to Clinical Implications" by G. Basta, 2008, Atherosclerosis, 196(1):9-21.

Once AGE binds to RAGE, downstream signaling pathways such as phosphatidylinositol-3-kinases (PI3K) and mitogen activated protein kinases (MAPK) become activated and lead to the translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [7, 9-13]. The activation of the transcription factor NF-κB leads to the production and release of inflammatory cytokines, growth factors, and adhesive molecules, which lead to the recruitment of immune cells [7, 9-13]. AGE activation of RAGE also leads to increased expression of RAGE itself, creating a positive feedback loop, further enhancing inflammation promoters [9, 11, 13]. Interaction with

RAGE also leads to the activation of nicotinamide adenine dinucleotide phosphate-oxidase (NAD(P)H), increasing intracellular oxidative stress, which in response leads to activation of NF-κB [7, 9-13]. The role of AGE signaling in inflammation has implicated this signaling pathway in the progression of a variety of disorders, including diabetes, Alzheimer's and cardiovascular disease [7, 9-14].

A pro-inflammatory environment is associated with promotion of tumorigenesis and a poor prognosis. Many cancers such as breast, prostate, colorectal and gastric have shown that AGE accumulation is increased and RAGE expression is upregulated in cancer tissue compared to normal [9, 13]. As discussed earlier AGE:RAGE signaling leads to activation of MAPK and PI3K pathways, both of which are known to be upregulated in cancer, promoting increased and uncontrolled growth of malignant tumors [9, 10]. Signaling through PI3K, MAPK and NADPH oxidase activation ultimately leads to the activation of NF-κB, as stated above [9, 11-13]. The resulting release of cytokines and growth factors, promotes increased recruitment of immune cells, increased oxidative stress and uncontrolled proliferation. Activation of NF-κB also leads to the increased expression of RAGE, leading to a feed-forward loop, further creating a pro-inflammatory and pro-oxidative microenvironment and a pro-tumorigenic environment in the presence of excessive AGEs (Figure 4) [9, 11-13, 15, 16].

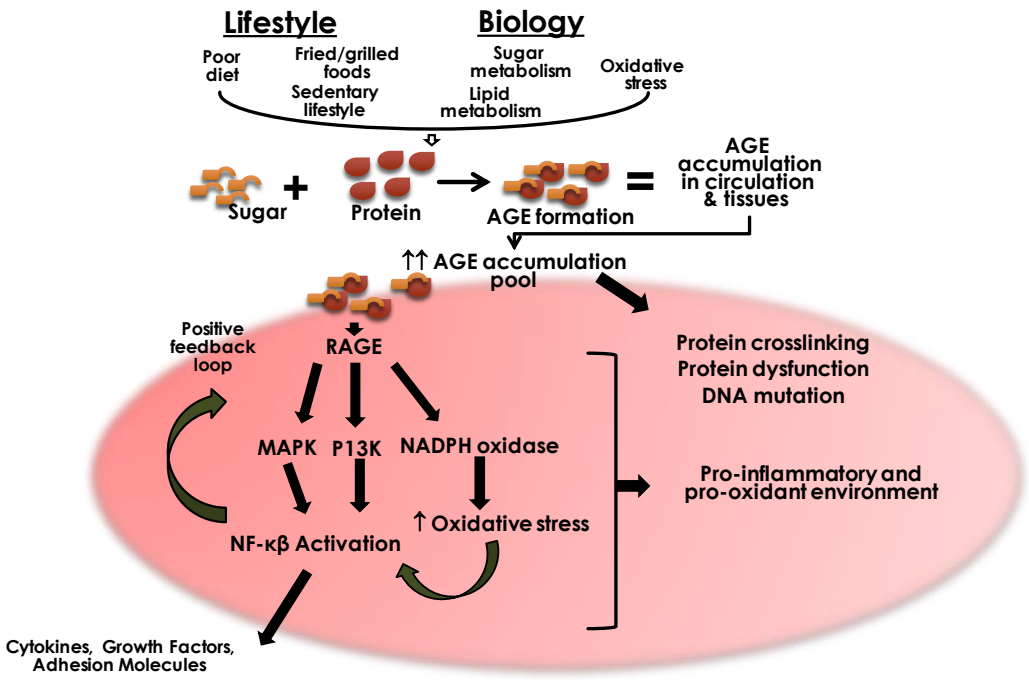


Figure 4. Schematic of AGE:RAGE Signaling. Lifestyle and biological AGEs bind to RAGE; PI3K, MAPK and NAD(P)H oxidase are activated downstream causing translocation of NF-κB to the nucleus, resulting in enhanced transcription and subsequent release of inflammatory markers as well as oxidative stress. In excessive presence of AGEs, microenvironment becomes pro-inflammatory and pro-oxidant.

AGE and RAGE in Macrophages and Fibroblasts. Macrophages are cells of the innate immune system that play a crucial role in host defense and tissue development. AGE:RAGE signaling leads to the release of cytokines, other inflammatory markers and adhesion molecules (ex. IL-1β, TNF-α, iNOS, ICAM-1 and VCAM), leading to inflammatory recruitment to a site of infection or injury [7, 9-13, 16]. RAGE is a member of the pattern-recognition receptors (PRRs) and can be found on macrophages. RAGE ligands, including AGEs leads to activation of macrophages, causing secretion of inflammatory markers, toxins and more RAGE ligands, including AGEs [7, 9-13, 16]. The receptor is not only shown to play a role in recruitment, proliferation and activation of macrophages, but also polarization, or the expression/secretion of different functional

factors in response to signals from the microenvironment [7, 10]. AGE activation of the NF-κB pathway through RAGE in diabetes lead to the polarization of macrophages to a pro-inflammatory M1 phenotype, with increased expression of IL-6, IL-10, TNF-α, and iNOS [7, 10].

Oxidative/carbonyl stress leads to increased expression of RAGE and other inflammatory markers on periodontal fibroblasts leading to increased inflammatory response [13, 17, 18]. AGE signaling through RAGE on pancreatic fibroblasts led to the activation of NF-κB, leading to increased expression of CCL5, IL-6 and IL-1B genes [17, 18]. AGE exposure to fibroblasts leads to activation of matrix metalloproteinase-2 (MMP-2), collagen 1 expression and upregulation of TGF-β1, TNF-α and IL-8 cytokines [13, 17, 18]. TGF-β1 and AGE crosstalk was shown to stimulate collagen III expression, and TGF-β1 signaling alone resulted in stimulation of GM-CSF and IL-6 expression, together leading to pro-inflammatory signaling and regulation of ECM turnover [17, 18].

Mammary Gland Development.

The mammary gland is a complex secretory organ composed of: epithelial cells, adipocytes, endothelial cells, fibroblasts and immune cells [19-22]. The main function of the mammary gland or breast is to secrete milk for the nourishment of newborns. The gland fulfils its function of supplying adequate milk by forming an extensive and functional tree-like structure of branched ducts, filling the majority of the fat pad [19-22]. Mammary gland development consists of three main stages; embryonic, pubertal and reproductive. Most of the extensive ductal-tree development occurs during adolescence [19-22]. While there are some hormonal and architectural differences between human and mouse mammary glands, researchers use the mouse as a model system to investigate the mechanisms of development as many models are implemented in breast cancer development studies as well [19-22].

Pubertal Mammary Gland Development. Pubertal development of the mammary gland begins during an extensive hormone-dependent phase of growth associated with increased epithelial cell proliferation [19-21]. This stage is associated with the formation of TEBs, highly proliferative club-like structures at the ends of ducts [23-25]. These bulbous structures form around 3 weeks of age and begin to penetrate the fat pad via the proliferation of a single layer of cap cells at the ends of the TEBs and the underlying epithelium [23-25]. Cap cells then differentiate into the myoepithelial layer surrounding the tubular ductal bilayer as the tree pushes into the mammary fat pad [21-25]. Proliferation is regulated by growth hormone (GH) inducing the expression of insulin-like growth factor-1 (IGF1) in the liver and mammary stromal cells. Acting together with estrogen (E_2) from the ovary, IGF1 helps to induce epithelial proliferation. Acting via a paracrine fashion E_2 signals through its receptor, estrogen receptor 1 (ESR1), to stimulate the release of amphiregulin (AREG), an epidermal growth factor (EGF) family member [21, 22]. AREG then binds to its receptor on stromal cells, inducing the release of fibroblasts growth factors (FGFs), which stimulate ESR1- luminal cell proliferation. Factors such as: TGF-β1, Reelin, Slit2 and Netrin1 leading to either positively or negatively regulate cell proliferation (Figure 5) [21, 22].

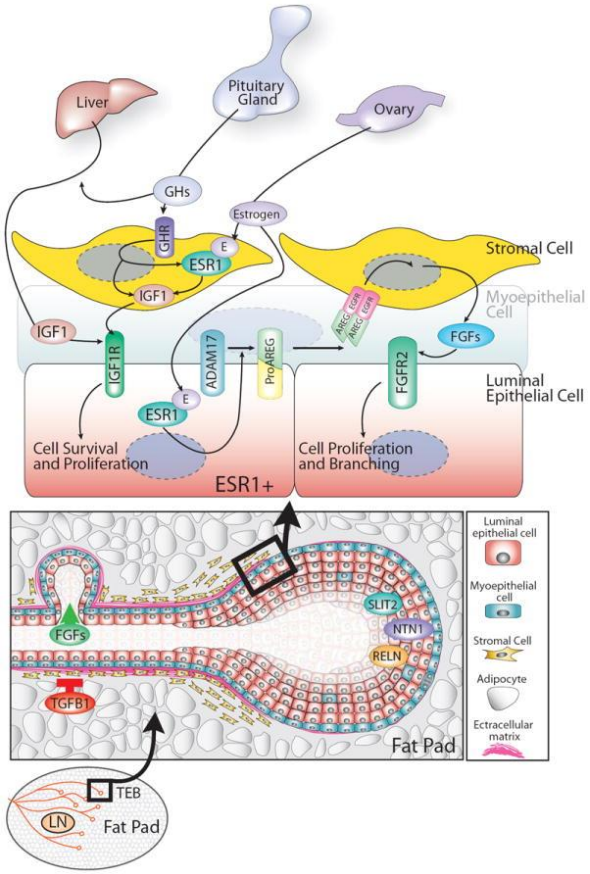


Figure 5. Hormonal Signaling During Pubertal Development. Signaling pathways during pubertal development leading to cell proliferation and survival of luminal epithelial cells of the TEBs leading to development of the ductal tree. Taken from "Mammary Gland Development" by H. Macias and L. Hink, 2012, Wiley Interdisciplinary Reviews. Developmental Biology, 1(4):533-57.

Extensive primary duct networks develop via the bifurcation of the TEBs, followed by branching of secondary side branches. Secondary branching from primary ducts continues until the ductal-tree fills about 60% of the fat pad, leaving space for the influence of pregnancy hormones. The mature ductal tree undergoes further branching under the influence of cycling ovarian hormones, leading to the formation of short tertiary side-branches [19-22]. Once the gland is fully formed at the end of pubertal

development, highly proliferative TEBs begin to taper off and become quiescent, forming terminal end ducts (TEDs) in adulthood (Figure 6) [23-25].

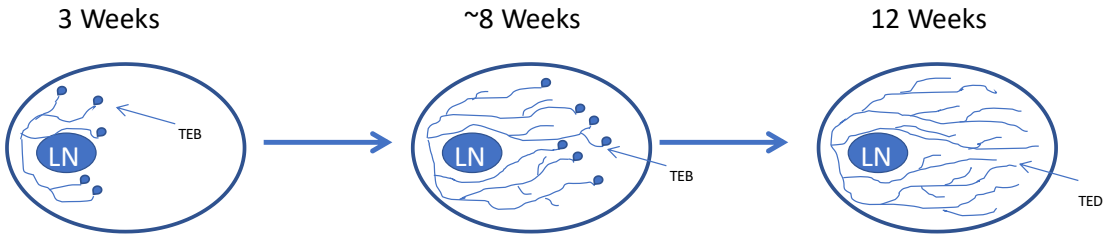


Figure 6. Pubertal Mammary Gland Development. Schematic view of pubertal mammary gland development from initiation (~3 weeks), to end of puberty (~8 weeks), to virgin adulthood (12 weeks).

Macrophages/Fibroblasts in Pubertal Mammary Gland Development.

Macrophages play a crucial role in correct tissue development of the mammary gland during puberty [19, 20]. During development, macrophages can normally be found around the TEBs, specifically around the neck and inside the TEBs, poised to be clearing cellular debris from cells undergoing apoptosis during development (Figure 7) [26-32]. Macrophages in resident tissue respond to cytokines in the tissue microenvironment during development. Cytokines such as IL-4 and IL-13 have been shown to be critical for differentiation of luminal epithelial cells [26-32]. It is believed that macrophages recruited to the mammary structures during development release factors for epithelial growth, angiogenesis or matrix remodeling, but also consumption of dead cells from turnover during development [26-32].

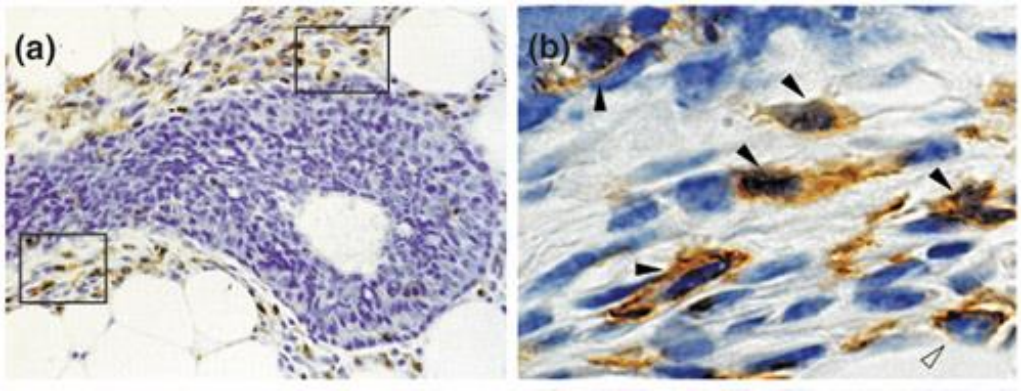


Figure 7. Macrophage Recruitment to TEBs. Anti- F4/80 staining of a TEB showing the localization of macrophages to the neck of TEBs (A), zoomed in field of view (B). Taken from "Requirement of macrophages and eosinophils and their cytokines/chemokines for mammary gland development." by V. Gouon-Evans, EY. Lin and JW. Pollard, 2002, Breast Cancer Research, 4(4):155-64.

To show the importance of macrophages in the developing gland, Gouon-Evans et al subjected mice to gamma-irradiation leading to depletion of circulating and mammary tissue leukocytes. Removal of the leukocytes led to impeded ductal development and absence of TEBs at the end of ducts, an overall neonatal look compared to control (Figure 8, A & D). When mice were subjected to bone marrow transplants, restoration of ductal outgrowth occurred, and the gland appeared more pubertal, compared to mice that were not subjected to transplants (Figure 8, C & D) [28, 29].

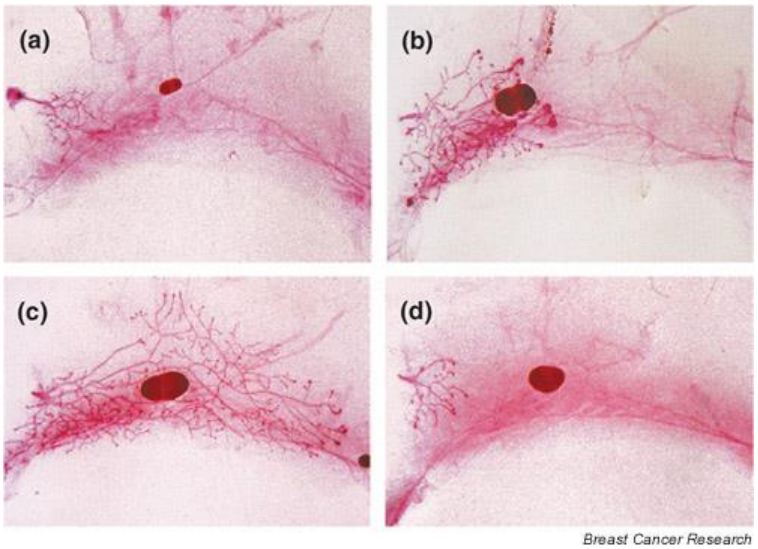


Figure 8. Importance of Macrophages in Development. Representative whole mounts of irradiated mice **(A)** compared to control **(B)**. As well as representative images of mice treated with bone marrow transplant **(C)** or not **(D).** Taken from "Requirement of macrophages and eosinophils and their cytokines/chemokines for mammary gland development." by V. Gouon-Evans, EY. Lin and JW. Pollard, 2002, Breast Cancer Research. 4(4):155-64.

The colony-stimulating factor 1 receptor (CSF-1R) is expressed on macrophages in mouse and human mammary glands during development [28, 29, 33-35]. Macrophage colony-stimulating factor 1 (CSF-1), is a growth factor that regulates proliferation, recruitment and survival of macrophages as well as other cells of the mononuclear phagocytic lineage. Mice with null mutations in CSF1 show a significant reduction of macrophages in the mammary gland. The reduction correlated with a delay and

reduction in TEB formation as well as reduced ductal morphogenesis. This phenotype was rescued when the mice were treated with human CSF1 (Figure 9) [33-35].

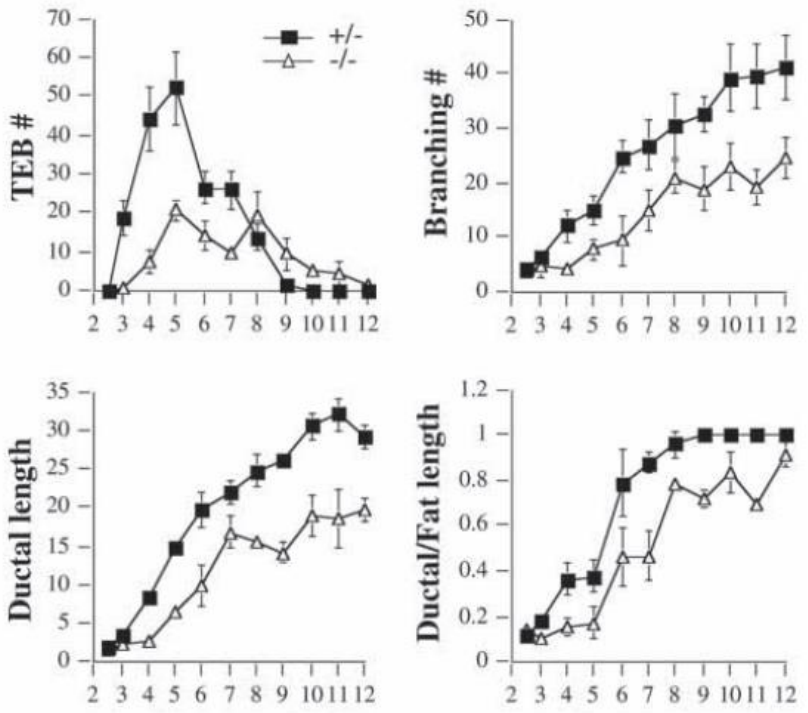


Figure 9. Importance of Macrophages in Development. TEB number, branching number, ductal length and the relative ductal growth in the mammary glands of control (squares) and experimental (triangles) mice. Showing significant effect on pubertal development in mice lacking macrophages. Taken from “Postnatal mammary gland development requires macrophages and eosinophils.” by V. Gouon-Evans, M. Rothenberg and JW. Pollard, 2000, Development, 127(11):2269-82.

Stromal fibroblasts are located throughout the fat pad and within proximity of the epithelial branching tree. Fibroblasts take part in bi-directional communication during branching morphogenesis by the release of growth factors, proteases, cytokines and other factors, playing an important role in epithelial cell survival and morphogenesis [19, 21, 31]. Studies have shown that fibroblasts play an important role in mammary ECM,

synthesizing such components as collagen, proteoglycans, fibronectin, as well as MMPs and other enzymes important for the degradation and regulation of the ECM [31].

Diet and Pubertal Mammary Gland Development. Mouse models of pubertal mammary gland development have shown drastic effects on the tightly regulated process with exposure to various diets. One of the most studied, is the high fat diet. C57BL/6 mice fed a pubertal high fat diet showed reduction in duct length and number of TEBs, a sparse ductal tree, increased mammary adiposity and reduced mammary epithelial cell proliferation compared to control diet. High fat diet in C57BL/6 mice was also shown to reduce E_2 responsiveness, a key hormonal factor in the developing gland. When BALB/c mice were fed the pubertal high fat diet a similar morphological change was observed in the glands. However, BALB/c mice showed increased mammary epithelial cell proliferation and reduced E2 responsiveness with no change in adiposity of the gland [36, 37].

Western diets are known to consist of high n-6 polyunsaturated fatty acids (n-6 PUFA), while Asian diets are known to consist of high n-3 PUFA. Consumption of n-6 PUFA during pubertal development of FVB/N and C57BL/6 mice resulted in increased TEB number, branching, epithelial cell proliferation compared to n-3 PUFA, which showed increase apoptosis mainly in TEBs [38, 39]**.**

Breast Cancer.

A normal functioning mammary gland is responsible for milk production and nourishment for newborns after childbirth. Human mammary glands consist of lobules for milk production known as lobes, or thin tube shaped structures that lead from the lobes to the nipple [20]. Fat and fibrous tissue surrounds the lobes, as blood and lymphatic vessels course throughout the breast which play a role in normal function, as well as tumor

metastasis [20, 40, 41]. Breast cancer has been ranked as the most common and deadly cancer among women. Around 70% of all breast cancer cases are shown to initiate in the ductal structures [40, 41].

Ductal Carcinoma In-situ (DCIS). Normal ductal structure consists of an outer myoepithelial layer and a luminal epithelial layer. The structure is surrounded by ECM and various stromal cells, including fibroblasts and macrophages [31]. It is believed that breast cancer begins with subtle changes in ductal or lobular epithelium, the structures may then develop further and become malignant and ultimately metastatic. This can be seen in the slight changes of the organized bi-layer ductal epithelium beginning to exhibit changes such as atypia, hyperplasia and ductal occlusion. These highly proliferative atypical ductal structures are considered early stage DCIS that have yet to break through the basement membrane [40, 42]. DCIS is also characterized with changes in the ECM and stromal cell recruitment. Myoepithelial cells can be altered and decreased potentially because of signals coming from tumor epithelial and stromal cells. The number of stromal cells, such as fibroblasts, myofibroblasts, macrophages, and endothelial cells increase, and continue to increase as the tumor growth and aggression continues. These early lesions are considered a risk factor for development of invasive breast cancer or invasive ductal carcinoma (IDC) (Figure 10) [40, 42, 43]. About 60,000 cases a year or 20% of all breast cancer diagnoses in the United States are considered DCIS [41]. Interestingly, aberrant activation of NF-κB by transgenic mouse model showed that this known downstream factor of AGE:RAGE signaling leads to the formation of DCIS like structures, possibly by the increased and sustained immune response as result of its activation [15].

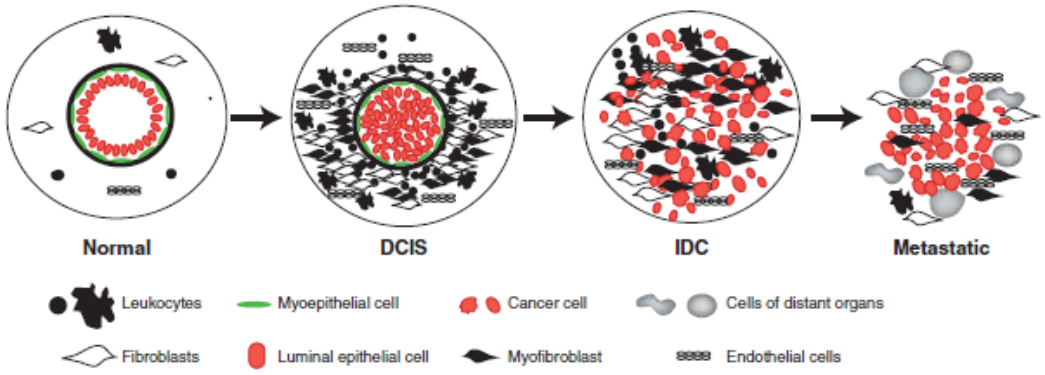


Figure 10. Progression of DCIS and Microenvironment Changes. Microenvironmental alterations during tumor progression from normal breast to DCIS to IDC and ultimately metastatic breast carcinoma. Taken from "The Role of the Microenvironment in Mammary Gland Development and Cancer" by K. Polyak and R. Kalluri, 2010. Cold Spring Harbor Perspectives in Biology, 2:a003244.

Macrophages/Fibroblasts in Breast Cancer. Macrophages not only play an important role in the developing gland, but also in the microenvironment of breast cancer during its progression [26, 27, 31, 44, 45]. Because of this, increased macrophage presence in biopsies has been linked to increased recurrence and decreased overall patient survival [41]. Classically activated macrophages or M1 macrophages are activated by cytokines such as interferon-γ, producing pro-inflammatory cytokines that aid in helper T-cell type 1 response. Tumor associated macrophages or TAMs are believed to be M2 macrophages or alternatively activated macrophages. They are activated by helper T-cell type 2 cytokines: IL-4, IL-10 and IL-13. TAMs secrete factors such as VEGF, EGF, FGFs and CXCL1/2 aiding in tumor progression and survival by inducing angiogenesis, cell survival and migration [40, 44, 45]. These macrophages have also been shown to interact with the ECM releasing pro-tumorigenic factors which suppresses $CD4^+$ and $CD8^+$ T lymphocytes anti-tumor function. Production of these

factors and changes to the ECM have also shown to promote breast cancer resistance to certain chemotherapeutic drugs [40, 44, 45].

Fibroblasts are the most abundant stromal cells in the mammary gland and play an important role in regulation of ECM during development [31]. Fibroblasts have also been shown to play an important role in cancer, called cancer-associated fibroblasts (CAFs) [40, 45, 46]. CAFs are similar to activated fibroblasts in wound healing, commonly associated with increased expression of α-SMA, the ED-A splice of fibronectin and other markers. Origins of CAFs are believed to be from activated fibroblasts, bone marrow derived mesenchymal stem cells (MSCs) or from cancer cells that undergo epithelial-mesenchymal transition (EMT) [40, 45-47]. CAFs have been shown to promote tumor onset and progression in a variety of ways, including, effects on E_2 levels, secretion of factors (HGF, TGF-β, SDF-1, VEGF, IL-6, MMPs, etc.), inducing stemness, EMT and epigenetic modifications. Interestingly, CAFs are induce invasion and metastasis in breast cancer [45, 47, 48]. In DCIS, CAFs induce the invasive capabilities of epithelial cells *in vitro* and *in vivo* through increased MMP14 expression and MMP9 activity [45, 46]. CAFs, with the aid of other stromal cells, also induce EMT changes in mammary gland epithelial cells, angiogenesis and macrophage recruitment [45, 47, 48].

Windows of Susceptibility. The 'lifecycle' of mammary gland development can be divided into five windows of cancer susceptibility: *in utero*, pubertal, pregnancy, postpartum involution and age-related involution. Each of these windows is limited in duration, making the at-risk populations identifiable (Figure 11) [49, 50]. A key characteristic of these risk 'windows' is tightly regulated tissue remodeling driven by hormonal signaling, as well as mammary epithelium and stromal cross-talk [51, 52]. Epidemiological studies have established a connection between life events and breast

cancer risk: age at puberty, age at first birth, length of time between menarche and first birth, and age at menopause [49]. These 'windows' and epidemiological studies led to the idea of preventative strategies for cancer prevention at crucial points during mammary gland development [49, 50, 53]. While intervention during pubertal development would have potential to dramatically reduce breast cancer incidence, treating adolescents with chemoprevention drugs is highly controversial, leaving preventative strategies such as diet, exercise and other lifestyle modifications [49, 50, 53].

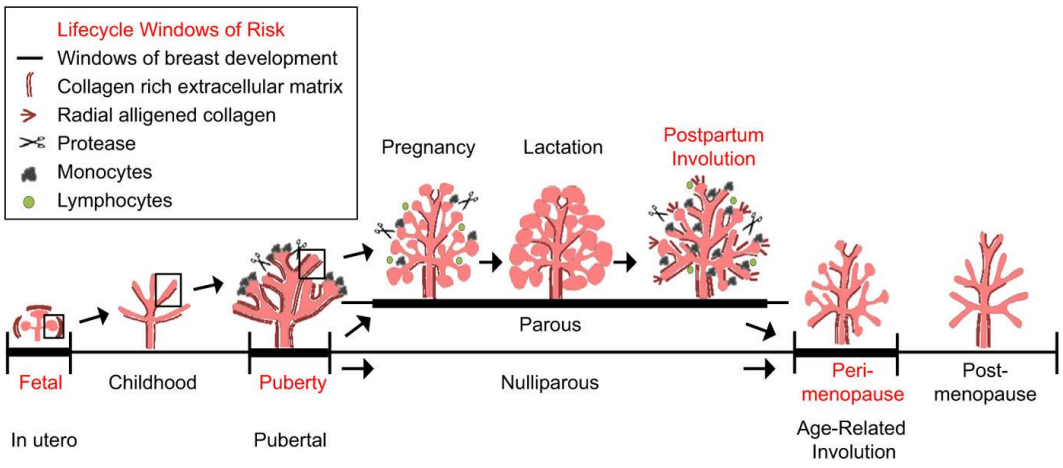


Figure 11. Windows of Susceptibility in Development. Schematic of breast development, showing 'windows of risk'. Taken from "Developmental windows of breast cancer risk provide opportunities for targeted chemoprevention." by H. Martinson, T. Lyons, E. Giles, V. Borges and P. Schedin, 2013, Experimental Cell Research, 319(11):1671-8.

Pubertal Diet and Breast Cancer Risk. Dietary factors are known to effect mammary gland development during puberty, tumor occurrence and progression [12, 37, 54]. In a BALB/c mouse model, mice were fed a high-fat diet during puberty, followed by initiation of tumorigenesis by 7,12-dimethylbenz[a]anthracene (DMBA). It was observed that mice fed a high-fat diet, regardless of intervention with low-fat diet during puberty,

showed increased tumorigenesis, with basal-like, triple-negative, adenosquamous characteristics. Tumors showed elevated levels of proliferation, macrophages and vascularization. Interestingly, these functional characteristics were observed before the activation of tumorigenesis in the developing mammary gland [55, 56]. Pubertal diet high in n-6 PUFA a component seen in modern Western Diet has shown to lead to changes in mammary gland development and mammary gland tumor development [36-39]. Studies done in rats have shown that a pubertal diet high in n-3 PUFA also leads to increased breast cancer risk, via increased proliferation, lipid peroxidation, DNA damage and cyclin D1 expression, as well as reduced apoptosis and BRCA1 expression [36-39].

Studies have also shown that pubertal exposure to phytoestrogen, genistein, reduces incidence of tumors in animal models, possibly via alteration of apoptosis/proliferation, reduction of TEB number, increased epithelial differentiation or upregulation of BRCA1 [54]. Rats fed a low-fat n-3 PUFA diet reduced mammary tumorigenesis associated with decreased proliferation, increased cell death and alteration of gene expression [36-39]. Exposure to 10% Flaxseed and optimal vitamin A levels were also shown to reduce breast cancer risk later in life [54]. Studies such as these show an important role of dietary exposure during pubertal mammary gland development with respect to breast cancer risk in adulthood.

Research Plan

Overall Rationale: Increased consumption of processed food, smoking and a sedentary lifestyle can lead to the accumulation of AGEs in the body. These reactive sugar metabolites are formed as a consequence of sugar metabolism and heat treatment of protein rich foods containing sugars and/or lipids. AGE:RAGE signaling has been shown to lead to the generation of reactive oxygen species (ROS) and activation of the PI3K/Akt and MAPK pathways, ultimately leading to the downstream activation of transcription factors NF-κB, hypoxia-inducible factor 1-alpha (Hif1alpha) and signal transducer and activator of transcription 3 (Stat3). This activation leads to an increase in cytokines, such as IL-6 and IL-1, E-selectin, vascular endothelial growth factor (VEGF), vascular cell adhesion protein 1 (VCAM-1), increased immune cell recruitment and further AGE production [1-5, 9, 10, 13, 18]. Studies have shown that transgenic mouse models leading to a reduction of macrophages in the mammary gland, resulted in the reduction of the number of terminal end buds (TEBs), decreased branching and caused abnormal TEB morphology. When the mice were treated to recover macrophage recruitment to the gland, TEB number and TEB morphology was rescued and ductal branching was increased, but not completely restored [19, 26, 28, 29, 31-35, 45]. Therefore, we hypothesize that chronic inflammation as a result of AGE:RAGE signaling may lead to phenotypic changes in pubertal mammary gland development leading to an increase in breast cancer risk.

Critical events during sensitive stages of development, such as puberty may influence the mammary gland microenvironment leading to increased breast cancer risk and/or promote tumor growth. Obesity, exercise, time of first child, breast feeding and birth control usage are important risk factors of breast cancer risk later in life. Diet and other lifestyle factors such as smoking and alcohol consumption have also been shown

to lead to increased breast cancer risk. For instance, Asian women typically have a lower risk of breast cancer compared to women exposed to Western diet, however within one generation of immigration to the United States Asian women have increased breast cancer risk, similar to that of women exposed to a Western diet for the majority of their lifetime [50, 51, 53, 57, 58]. The increase of AGE presence in the modern world from highly processed food and a sedentary lifestyle, with the consequence of its signaling through RAGE has led us to investigate its effects on mammary gland pubertal development and breast cancer risk in adulthood.

Hypothesis: We hypothesize that mice fed a high AGE diet will result in dysregulation of normal pubertal mammary gland development, leading to an increase in breast cancer risk.

Specific Aim 1: To test the hypothesis that high AGE diet impairs normal pubertal mammary gland development. Pubertal mammary gland development is a tightly regulated process of cellular communication between stromal and epithelial cells. Immune cells such as macrophages and eosinophils play an important role in the development of the gland as seen in knockout mouse models. Reduction of immune cells leads to alterations of ductal morphogenesis and TEB number in the gland during puberty [28, 29]. AGE:RAGE signaling leads to a pro-oxidant and pro-inflammatory environment [11]. Transgenic mice with aberrant activation of NF-κB during development have shown phenotypic changes of mammary glands associated with increased branching and TEB size, possibly from inflammatory factors [15]. NF-κB is a known downstream transcription factor of AGE:RAGE signaling [11]. These observed changes lead us to develop a model of dietary exposure during pubertal development of high AGE to determine the effects these reactive sugar metabolites may cause on pubertal mammary gland development.

- **Task 1:** Establish the mouse model for regular, low (endogenous) AGE and high (exogenous) AGE diets:
    1) Assess AGE levels in food of mouse diets by Dot Blot and ELISA
    2) Assess circulating AGE levels in dietary mouse models by ELISA
    3) Assess AGE accumulation in mammary gland tissue of dietary mouse models by immunohistochemistry
- **Task 2:** Evaluate the gross mammary gland phenotype differences among the diets in pubertal development using carmine stained whole mounts:
    1) Quantify TEB size
    2) Quantify TEB number
    3) Quantify ductal branching
    4) Quantify ductal extension
- **Task 3:** Evaluate the morphological differences of pubertal structures among the diets:
    1) Histologically evaluate TEB morphology
    2) Histologically evaluate ductal structures
    3) Histologically evaluate aberrant stromal recruitment surrounding TEBs and ductal structures
- **Task 4:** Evaluate expression of RAGE and in dietary mouse models
    1) Perform RAGE IHC on mammary gland tissue

Experimental Design

AGE Specific Diets. The diets that we utilized in our development model are referred to as: regular, low AGE and high AGE. Our control diet or regular diet (TestDiet DIO 58G9) consists of 60% k/cal fat. The low AGE diet (TestDiet DIO 58G7), as named by our research team, consists of 12% k/cal fat. The experimental diet or high AGE diet is the

low AGE diet autoclaved at 120ºC for 15 minutes to promote AGE formation. Additional nutritional information of the specific diets is provided in Table 1. Each week a measured amount of food was supplied to the mice ad lib and then measured the following week to ascertain the amount of food consumed per mouse per week.

Table 1. **Nutritional Information of Control and Experimental Diets**

	Protein %	**Fat %**	**Fiber %**	**Carbohydrates %**
Regular	24.2	34.7	5.5	27.8
Low/High AGE*	20.8	23.6	5.8	41.2

*High AGE is the same as low, but autoclaved at 120ºC for 15 minutes

AGE Dot Blot. AGE content was qualitatively evaluated in the test diets and control diets via Dot Blot analysis. Regular food was autoclaved at 120ºC for 15 minutes as described above for the experimental diet to compare the AGE formation in control diet during autoclave process. Food (1 g) was dissolved in 10 mL of 1x PBS by rotation overnight. Carefully 2 µL of dissolved food was applied to a nitrocellulose membrane (Fisher Scientific, Fair Lawn, NJ), covered and left to dry at room temperature (RT) for 1 hour. The membrane was blocked with 5% BSA in 1X TBST for 30 minutes on a shaker at RT. Primary antibody for AGE (ab23722; Abcam, Cambridge, MA) was diluted 1:4000 using 2.5% BSA in 1X TBST and incubated overnight at 4°C on a shaker. The membrane was washed 5 times for 10 minutes in 1X TBST on an orbital shaker. Secondary anti-rabbit HRP linked antibody (Cell Biolabs, Inc. San Diego, CA) was applied to the membrane at a 1:2000 dilution for 2 hours at 4°C on a shaker. The membrane was washed 5 times for 10 minutes in 1X TBST on an orbital shaker. Blots were then incubated with 2 mL Clarity Western ECL substrate (Pierce, Rockford, IL) for 5 minutes to activate the signal. Finally, using autoradiography film (Denville Scientific,

Metuchen, NJ) blots were developed at a one hour time exposure with an automatic developer (Kodak, Rochester, NY).

AGE ELISA. AGE concentration was quantitatively evaluated in the experimental and control diets using the OxiSelect™ Advanced Glycation End-Product (AGE) Competitive ELISA Kit (Cell Biolabs, Inc. San Diego, CA). AGE Conjugate solution was prepared, 100 µL added to each well of the 96 well-plate and incubated overnight at 4ºC. The solution was removed and washed twice with 1X PBS, 200 µL of Assay Diluent was added to each well and blocked for 1 hour at RT, and stored at 4ºC. A series of AGE-BSA standards was prepared, ranging from 1.56 µg/mL to 100 µg/mL. Food solutions were prepared by dissolving 1 g of each diet in 1x PBS. Assay Diluent was removed, and 50 µL of each unknown sample and standard was assayed in duplication on the AGE Conjugate coated plate. The plate was incubated at RT for 10 minutes on an orbital shaker. Anti-AGE Antibody (Cell Biolabs, Inc.) was diluted 1:1000 and 50 uL added to each well and incubated at RT on an orbital shaker for 1 hour. Wells were washed 3X times with 250 µL 1X Wash Buffer (Cell Biolabs, Inc.) with thorough aspirations in-between. Excess 1X Wash Buffer was removed and 100 µL of 1:1000 Secondary Antibody-Horseradish Peroxidase (HRP) Conjugate (Cell Biolabs, Inc.) was added to all wells and incubated for 1 hour on an orbital shaker at RT. Wells were washed 3X times with 250 µL 1X Wash Buffer. Substrate Solution (Cell Biolabs,) was warmed to room temperature and 100 µL was added to each well, watching carefully for color change in samples. Reaction was stopped when change in color was observed by adding 100 µL of Stop Solution (Cell Biolabs, Inc.). Results were read immediately on microplate reader (Bio-Tek Instruments, Inc. Winooski, VT) at 450 nm.

Development of Mouse Model. Dietary Mouse Model. Female wild-type FVB/n mice were weaned at 3 weeks of age, a baseline blood sample was collected via cardiac

puncture from a sub-set group. Specific diets were started at weaning; high AGE, low AGE and regular. Each week the weight of each mouse was recorded and analyzed for longitudinal analysis, as was food consumption. Diets continued till desired endpoints of 7, 8 (pubertal) and 12 weeks (mature virgin). At these endpoints, mammary glands were extracted from the mice for whole mounting and paraffin embedding, focusing on the inguinal gland, utilizing the lymph node as a geographical marker for analysis. Tissue was also collected and fresh frozen for future protein and RNA extraction. Blood was collected at each end-point via cardiac puncture (Figure 12).

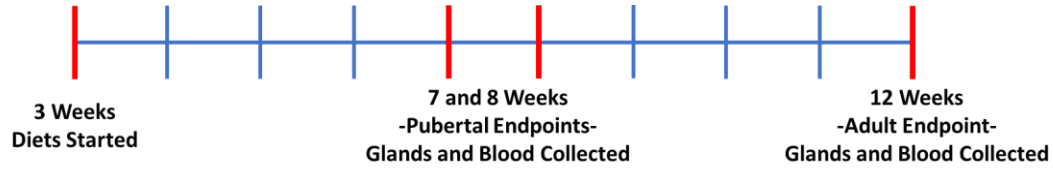


Figure 12. Timeline of Dietary Mouse Model.

Evaluation of Circulating AGEs. Endpoint blood samples were centrifuged at 6,000 RPM for 20 minutes to separate and isolate serum and stored at -80ºC. Samples from weaning and end-points (7, 8, and 12 weeks) were compared using Dot Blot analysis (as described above) to determine differences in circulating AGE levels before and after diet. Serum samples were diluted 1:20 and followed the previous mentioned protocol with a 5 second exposure time. Samples were also evaluated for circulating AGE concentration using OxiSelect™ Advanced Glycation End-Product (AGE) Competitive ELISA Kit (Cell Biolabs, Inc.), as described above.

Whole Mounts and Analysis. Extracted mammary glands were stretched onto a glass slide and fixed in 4% paraformaldehyde (PFA) at 4ºC overnight, followed by a series of washes to rehydrate the tissue: 1X 70% ethanol (EtOH), 1X 50% EtOH, 1X 30% EtOH

and autoclaved water for 1 hour each at RT. Tissue was then immersed in carmine staining solution (0.2% carmine, 0.5% aluminum potassium sulfate (Sigma-Aldrich Co., St. Louis, MO)) overnight at RT, followed by a series of washes to dehydrate the tissue: autoclaved water, 1X 30% EtOH, 1X 50% EtOH, 1X 70% EtOH,; 2X 95% EtOH, 2X 100% EtOH for 1 hour each. Glands were then covered with a clean slide and clipped together and immersed in xylene overnight. Glass cover slips were then mounted on top of slides using Permount® (Fisher Scientific, Fair Lawn, NJ). Once dry, the whole mounts were photographed using a photo scanner (Epson Perfection 2450 Photo).

TEB Area was assessed using ImageJ software and the scans of the inguinal glands. TEBs were identified as club-like structures at the end of branches and predominantly in the leading edge of the developing gland. After setting the scale, length (from tip to neck) and width (at the widest point) of the TEB was assessed as illustrated in Figure 13 A. The collected measurements were then placed into the formula for the area of an ellipse (πab) to calculate the area of the TEB. Average area was compared between individual mice and among the diets.

TEB Number was assessed using ImageJ software and the scans of the inguinal glands. A field of view 2 mm^2 was established and placed at the top edge of the gland with the leading edge centered. Once the number of TEBs was calculated per field, the field was moved down the gland till the entire gland was covered and counted (Figure 13 B). Average number of TEBs per field of view was compared between individual mice and among the diets.

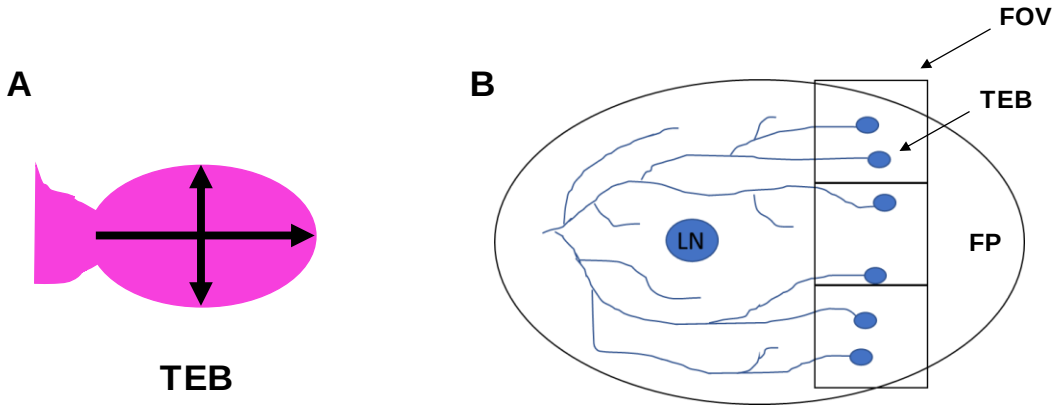


Figure 13. Schematic of TEB Size and TEB Number Analysis. (A) TEBs were measured at the widest point and from neck to tip to obtain the area using the formula for an ellipse. **(B)** TEB number, using inguinal whole mounts was analyzed by moving the designated FOV down the leading edge in the fat pad (FP).

Ductal Branching was assessed using ImageJ software and the scans of the inguinal glands. A field of view 2 mm^2 was established and placed at the top edge of the gland with the leading edge to the far-right side of the field. The field was then moved 2 fields back into the ductal tree of the gland. Primary (1º), secondary (2º) and tertiary (3º) branchpoints were counted as seen in Figure 14. Once all branchpoints per field were counted, the field was moved down the gland till the entire gland was covered and counted. Total branching was calculated by summing all branchpoints per field of view. Average branchpoints (1º, 2º, 3º, and total) were compared among the diets (Figure 14 B).

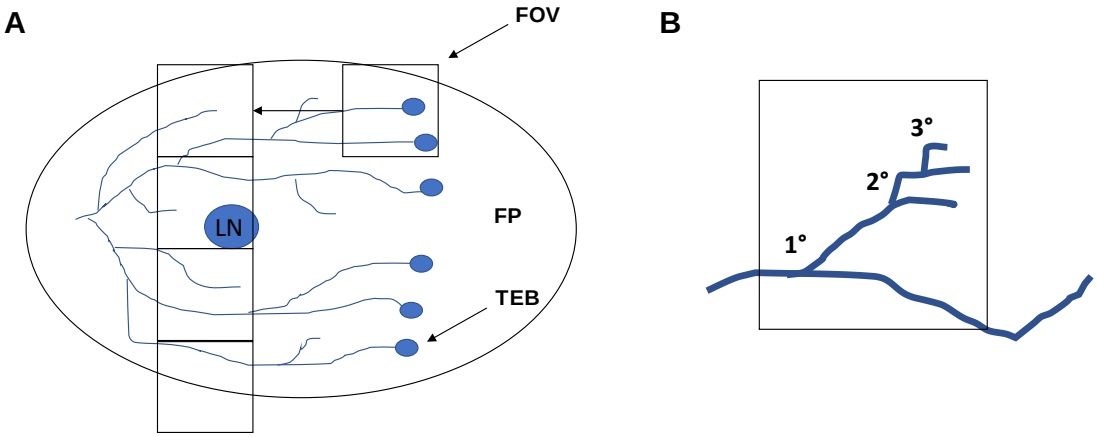


Figure 14. Schematic of Ductal Branching Analysis. (A) Schematic for the analysis of ductal branching using inguinal whole mounts. **(B)** Demonstration of primary (1°), secondary (2°) and tertiary (3°) branchpoints.

Ductal Extension was assessed using ImageJ software and the scans of the inguinal glands. After setting the scale, length from the end of the lymph node to the tip of the furthest TEB in the leading edge was measured (Figure 15). Average ductal extension was compared among the diets.

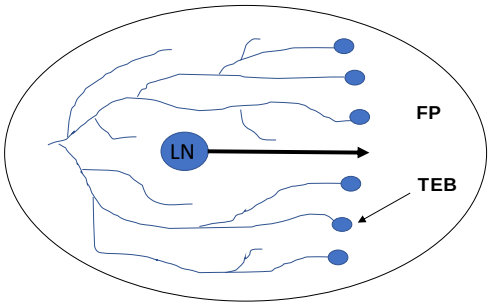


Figure 15. Schematic of Ductal Extension Analysis. Measurements were made from the lymph node (LN) to the tip of the furthest TEB.

Paraffin Embedding. After extraction, mammary glands were placed into 4% PFA at 4ºC overnight. Followed by a series of washes to dehydrate the tissue: 1X 70% EtOH for 1 hour; 2X 95% EtOH, 2X 100% EtOH for 1 hour each; toluene for 1-4 hours. Glands were then placed in 50/50 mix toluene/paraffin at 60ºC overnight. The following day, glands were incubated in paraffin 2 times for 2 hours each at 60ºC. Finally, glands were embedded in paraffin dorsal side down.

AGE/RAGE IHC. In order to assess AGE localization and RAGE presence in the mammary gland, paraffin embedded inguinal mammary glands were sliced 5 µm thick and utilized for IHC. Slides were deparaffinized at 60º C for 1 hour. Followed by a series of washes to rehydrate the tissue: 2X xylene for 20 minutes each; air dry for 15 minutes; 3X 100% EtOH, 2X 95% EtOH, 1X 70% EtOH, and 1X 50% EtOH for 10 minutes each; 3X diH_20 for 5 minutes each. For antigen retrieval Vector unmasking solution (Vector Labs, Burlingame, CA) was used at a 1:100 dilution. Slides were placed in a pap jar containing the diluted unmasking solution, brought to boiling and placed in a vegetable steamer containing the diluted solution, for 30 minutes at 90°C. The slides were then placed in a pap jar containing 0.3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ) for 30 minutes to remove any endogenous peroxidase, then washed in 1X phosphate-buffered saline containing 0.01% Tween-20 (PBST) (Fisher Scientific, Fair Lawn, NJ) for 5 minutes. Slides were then incubated in VECTASTAIN® horse serum (Vector Labs, Burlingame, CA) to block non-specific binding for 20 minutes at RT in a humidified chamber. Primary antibody (Abcam, Cambridge, MA) was diluted using 2.5% horse blocking serum diluted with 1X PBS and applied to slides to incubate overnight at 4°C in a humidified chamber (Table 2.). Primary antibody was then removed by washing 3 times in 1X PBS for 5 minutes on an orbital shaker. Followed by incubation with VECTASTAIN® secondary antibody solution (Vector Labs, Burlingame, CA) for 30 minutes at RT in a humidified chamber. Slides were washed again 3 times in 1X PBS for 5 minutes on an orbital shaker. Samples were then incubated with SIGMAFAST^{TM} DAB (Sigma-Aldrich Co., St. Louis, MO) to activate the signal, then placed in diH_20 3 times for 5 minutes to stop enzymatic reaction. Hematoxylin (Fisher Scientific, Fair Lawn, NJ) was placed on the tissue and placed into diH_20. Followed by a series of washes to complete the counter stain: HCl-EtOH for 90 seconds; NH_4OH-EtOH for 45 seconds; diH_20 for 3 minutes; Scott's Buffer for 1 minute. Stained slides were then dehydrated by a series of

washes: 3-diH_20 for three minutes each; 1X 50% EtOH, 1X 70% EtOH, 2X 95% EtOH, and 3X 100% EtOH for 5 minutes each; air dry for 15 minutes; 2X xylene for 10 minutes each. Glass cover slips were then mounted on top of slides using Permount® (Fisher Scientific, Fair Lawn, NJ). Imaging was completed using an Olympus DP80 microscope (Olympus American, Inc. Center Valley, PA).

Table 2. Antibody Information for Specific Aim 1 IHC Methodology

	Ab Number	Dilution	Secondary Ab	DAB	Hematoxylin
AGE	ab23722	1:50	Rabbit	30 sec	1 min
RAGE	ab37647	1:50	Rabbit	30 sec	1 min

H&E. In order to assess morphological changes in mammary glands among diets, paraffin embedded inguinal tissue was sliced 5 µm thick and utilized for H&E. Slides were deparaffinized at 60º C for 1 hour. Followed by a series of washes to rehydrate the tissue: 3X xylene, 3X 100% EtOH, 1X 95% EtOH, 1X 80% EtOH for 3 minutes each; diH_20 for 5 minutes. Samples were then covered with Hematoxylin (Fisher Scientific, Fair Lawn, NJ) for 3 minutes and rinsed in diH_20. Slides then followed a series of washes: tap water for 5 minutes; HCl-EtOH 8-12 dips; 2-tap H_20 for 1 minute; diH_20 for 2 minutes. Tissue was then covered by Eosin (Fisher Scientific, Fair Lawn, NJ) for 30 seconds. Followed by a series of washes to dehydrate the tissue: 3X 95% EtOH, 3X 100% EtOH for 5 minutes each, 3X xylene for 15 minutes each. Glass cover slips were then mounted on top of slides using Permount® (Fisher Scientific, Fair Lawn, NJ). Imaging was completed using an Olympus DP80 microscope (Olympus American, Inc. Center Valley, PA).

Results

High AGE food is an exogenous source of AGEs, low AGE food is an endogenous source of AGEs. In order to determine AGE content in the experimental diets, we utilized ELISA and Dot Blot analysis. The high AGE diet contained a significant amount of pre-formed AGEs from the autoclaving process when compared to low AGE and control diets (Figure 16). By dot blot analysis, the regular diet showed no detectable pre-formed AGEs, even when autoclaved in the same manner as high AGE food (Figure 16 B). Low AGE while containing some pre-formed AGEs still contains the building blocks for AGE formation, i.e. increased sugar content (Table 1), therefore we predict that we may observe changes in mammary gland development with the low AGE diet when compared to the regular diet, but not as severe as those observed in mice fed the high AGE diet.

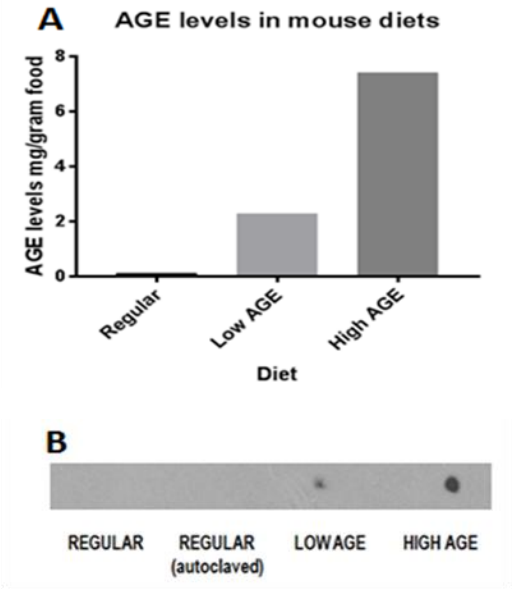


Figure 16. AGE Content in Mice Diets. ELISA **(A)** and Dot Blot analysis **(B)** for AGE content in diets used in our model reveal significant amount of pre-formed AGEs in the high AGE diet compared to control diets.

High AGE and low AGE diet lead to an increase in circulating AGEs. To assess the amount of circulating AGEs in mice fed the regular and experimental diets we utilized ELISA analysis. High AGE saw an increase in circulating AGE levels compared to regular and pre-draw, low AGE saw an increase in circulating AGE levels compared to

baseline levels, further supporting the idea of exogenous and endogenous AGEs (Figure 17). In all three endpoints both diets showed to have more circulating AGEs compared to regular diet fed mice.

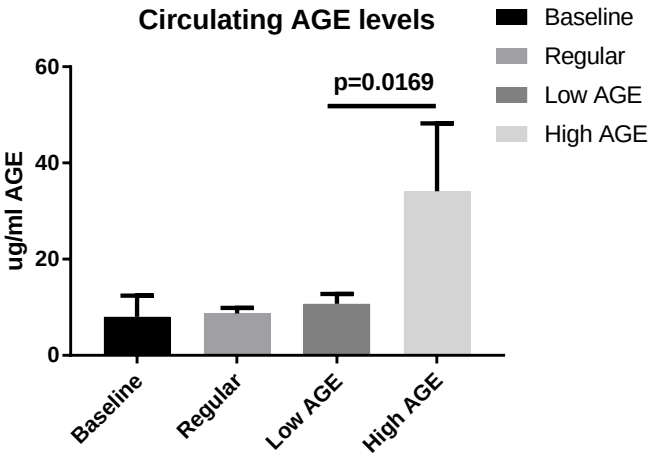


Figure 17. Pubertal Circulating AGE Levels in Mice. Circulating AGE levels evaluated via ELISA revealed significant increase of AGEs in the high AGE fed mice at the 8 week timepoint.

Diets did not result in significant changes in weight or food consumption. We monitored weight gain and food consumption among the diets, as obesity and over consumption of diets caused by enhanced taste may lead to changes in development and AGE content in the mice. Each week food consumption and weight was monitored to assess changes. The weight of the mice showed there was no overall significant difference among the diets (Figure 18 A). Monitoring these variables was important to ensure that any effects observed were not caused by external factors such as obesity, as obesity has been shown to lead to changes in development and increase in breast cancer risk [52, 54]. Food consumption was monitored to assure that mice were not consuming more of one diet compared to another, leading to a more dramatic

phenotype. Food consumption, while showing fluctuations from week to week, showed no overall significant difference among the models (Figure 18 B).

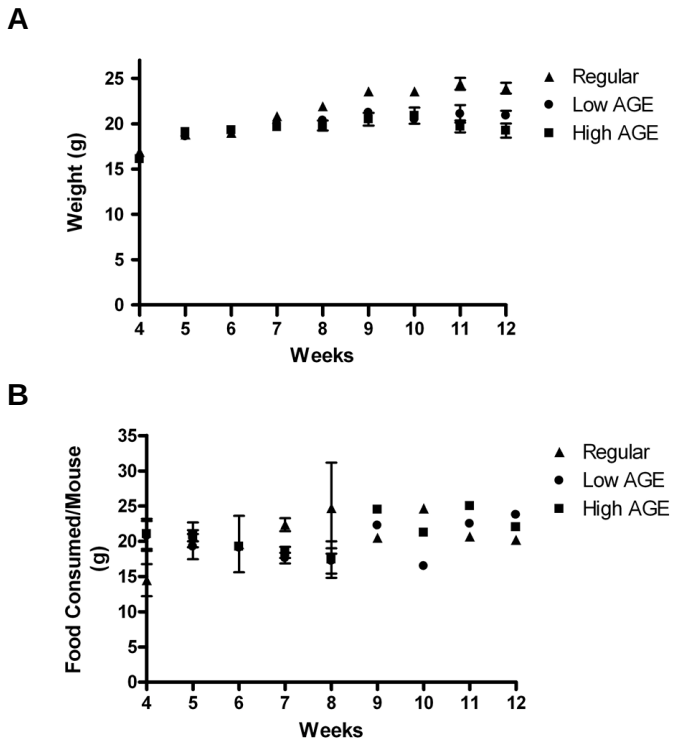


Figure 18. Mice Weight and Food Consumption. Longitudinal analysis shows there is no significant difference in weight **(A)** and food consumption **(B)** per mouse among the diets.

High AGE diet resulted in increased TEB size and number in mice during pubertal development. Macroscopically looking at whole mounts of High AGE diet we observed increased TEB size and number compared to other glands. This same phenotype was observed in transgenic mice with aberrant NF-κB activation, a known downstream target of AGE:RAGE signaling [15]. This observation prompted us to quantify changes in TEB size and number during pubertal development and adulthood using whole mounted inguinal mammary glands. The results show that mice fed a high AGE diet had a significant increase in TEB area compared to low AGE (7 weeks) and regular diets (7 &

8 weeks). Interestingly, low AGE also showed an increase in TEB area compared to regular diet at week 7 (Figure 19 A). Increased number of TEBs was also seen at both 7 and 8 weeks using carmine stained glands of mice fed the high AGE diet compared to regular diet (Figure 19 B). This observation was carried into adulthood as presence of TEBs was seen in both the high and low AGE diets, a time point where TEBs are commonly regressed to TEDs (Table 3).

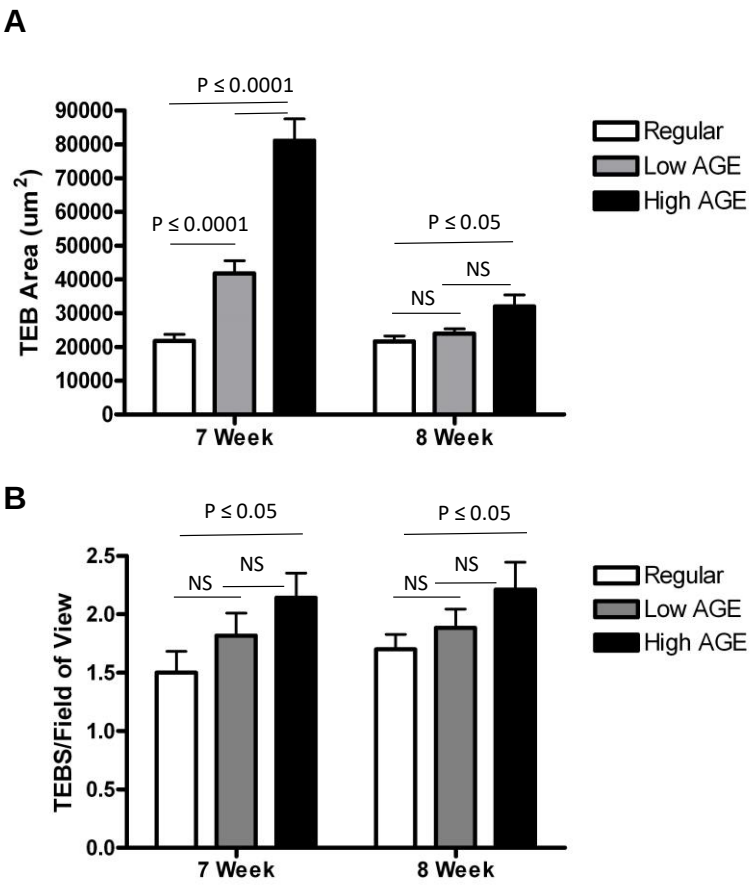


Figure 19. TEB Area and Number Among Diets. Carmine stained whole mount analysis revealed that a diet high in AGEs resulted in increased TEB area **(A)** as well as number **(B)** per field of view.

Table 3. Number of Inguinal Glands that Contained TEBs at Adulthood per Diet.			
	Regular	**Low AGE**	**High AGE**
Glands with TEBs	2	5	4
Total Glands	4	5	4
Percent (%)	50%	100%	100%

Mice fed a high AGE diet resulted in increased ductal branching. Macroscopically looking at whole mounts of high AGE diet we observed what looked to be increased thickness of the ductal tree compared to other glands. This was a phenotype also caused by increased branching in transgenic mice with aberrant NF-κB activation, a known activated transcription factor of AGE:RAGE signaling [15]. This observation prompted us to quantify changes in ductal branching during pubertal development and adulthood utilizing whole mounted inguinal mammary glands. We observed that mice fed a high AGE diet showed significant increase in total ductal branching per field of view at 7, 8 and 12 weeks (Figure 20 A). Looking specifically at branchpoints in these glands, we see that at 8 and 12 weeks a diet high in AGEs leads to increased primary branches per field of view compared to regular and low AGE diets (Figure 20 B). Secondary and tertiary branching showed no significant difference among the diets (Figure 20 C & D).

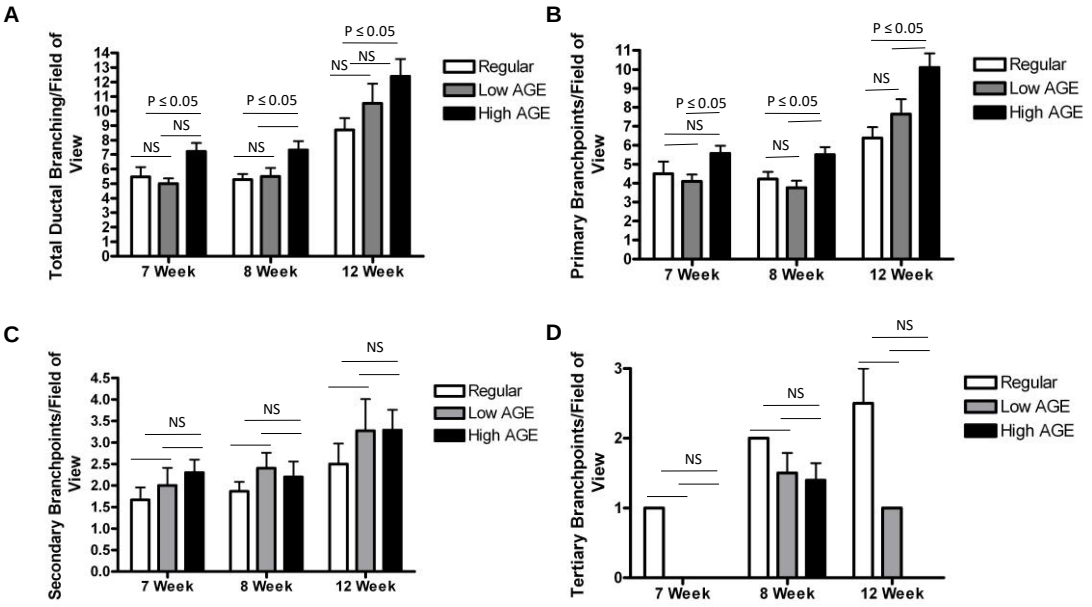


Figure 20. Ductal Branching Among Diets. Carmine stained whole mount analysis revealed that a diet high in AGEs resulted in increased total ductal branching **(A)** per field of view, mostly as a result from increased primary branching **(B)** per field of view. No significant difference was observed among secondary **(C)** and tertiary branching **(D).**

Ductal extension was delayed in mice fed a high AGE diet and recovered at adulthood. Macroscopic examination of high AGE diet whole mounts showed a decrease of ductal tree extension into the mammary fat pad compared to other inguinal mammary glands. This observation prompted us to quantify changes in ductal extension during pubertal development and adulthood by utilizing whole mount inguinal glands. We observed a significant reduction of ductal extension in mice fed a high AGE diet during pubertal development. However, this phenotype was recovered in adulthood (Figure 21).

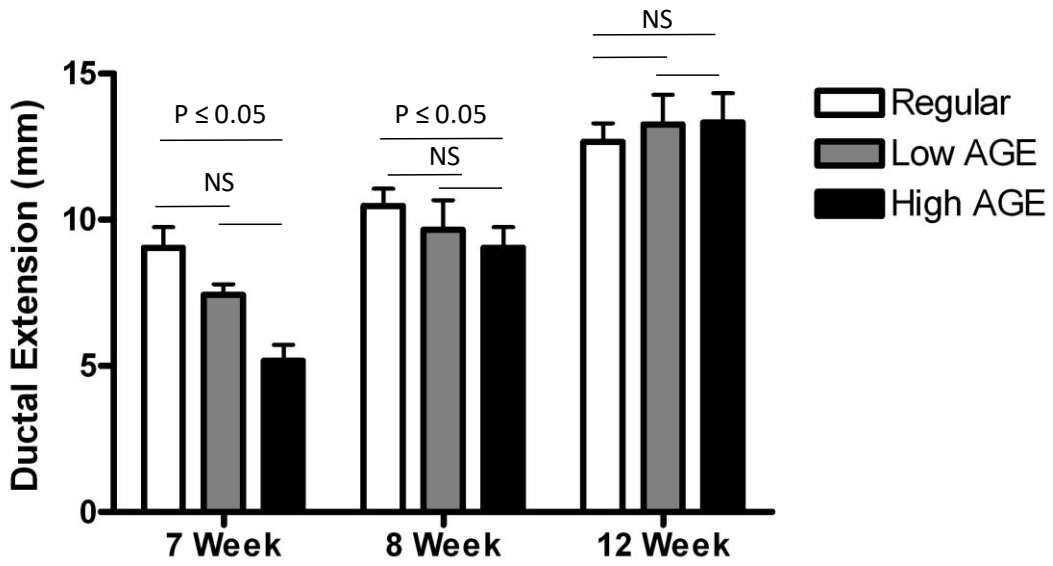


Figure 21. Ductal Extension Among Diets. Carmine stained whole mount analysis revealed that a diet high in AGEs resulted in a delay in ductal extension during puberty which recovered in adulthood.

TEB and duct morphology was disorganized in mice fed a high AGE diet.

The increased size of TEBs led us to propose that the high AGE diet may cause morphological changes in pubertal structures. In order to assess these changes in TEB and duct morphology we utilized H&E analysis of pubertal time points using inguinal mammary glands. Histological analysis of H&E stained glands showed a disorganization in TEB morphology from their normal club-like structures, mice fed a high AGE and low AGE diet showed a thickening of TEB

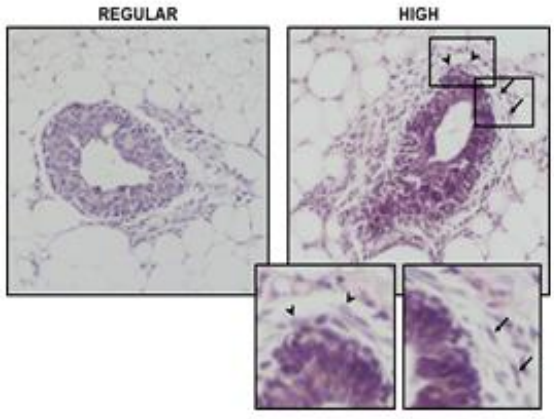


Figure 22. Morphological Changes of TEBs. Representative H&E stained glands showed that high/low AGE diet TEBs were surrounded by a thicker more cellular stroma compared to regular. Consisting of macrophages (arrowheads) and fibroblasts (arrows).

epithelial at the tail and reduced cellular presence at the tip. The diets also resulted in changes in stroma surrounding the TEBs. Normal TEB stromal surrounds the neck of the TEB, in mice fed a high/low AGE diet increased stroma was localized around the entire structure and appears thicker and more cellular. We had our collaborating veterinarian pathologist (Dr. Kristi Helke) examine the stroma surrounding the TEBs and confirmed by morphological analysis that the predominant cell type recruited to the abnormal stroma to be macrophages and fibroblasts (Figure 22). H&E stained mammary glands showed that both diets also resulted in a thickening of ducts from their normal epithelial mono-layer to multi-layered structures with a thickening stroma, as seen around the TEBs (Figure 23). Ducts of both high and low AGE diet also demonstrated sloughing of the epithelial cells into the lumen during puberty and adulthood compared to regular diet (Figure 23 C).

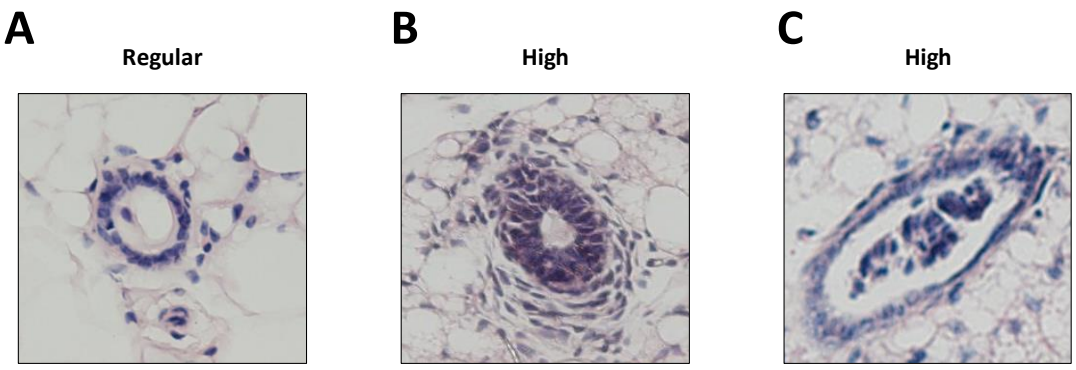


Figure 23. Morphological Changes of Ducts. Representative H&E stained glands showed that high **(B)** and low AGE ducts were multi-cellular and surrounded by a thicker more cellular stroma compared to regular **(A)**. High **(C)** and low AGE ducts also showed sloughing of epithelial cells into the lumen.

High AGE diet led to increased RAGE and AGE presence in the developing gland. A result of AGE:RAGE signaling is a positive feedback loop leading to increased RAGE expression prompting us to assess changes in RAGE and tissue specific AGE presence

of structures during pubertal development via IHC analysis using inguinal glands. Regular diet mice TEBs and ducts epithelial cells stained positive for RAGE, when the mice were subjected to high and low AGE diets an upregulation of positively stained epithelial cells in these structures were observed (Figure 24). Little to no positive stained adipocytes and stromal cells were observed in regular diet mice, when a pubertal high AGE diet was sustained, increased RAGE staining was observed in cells surrounding TEBs and ducts, suspected to be macrophages (Figure 24).

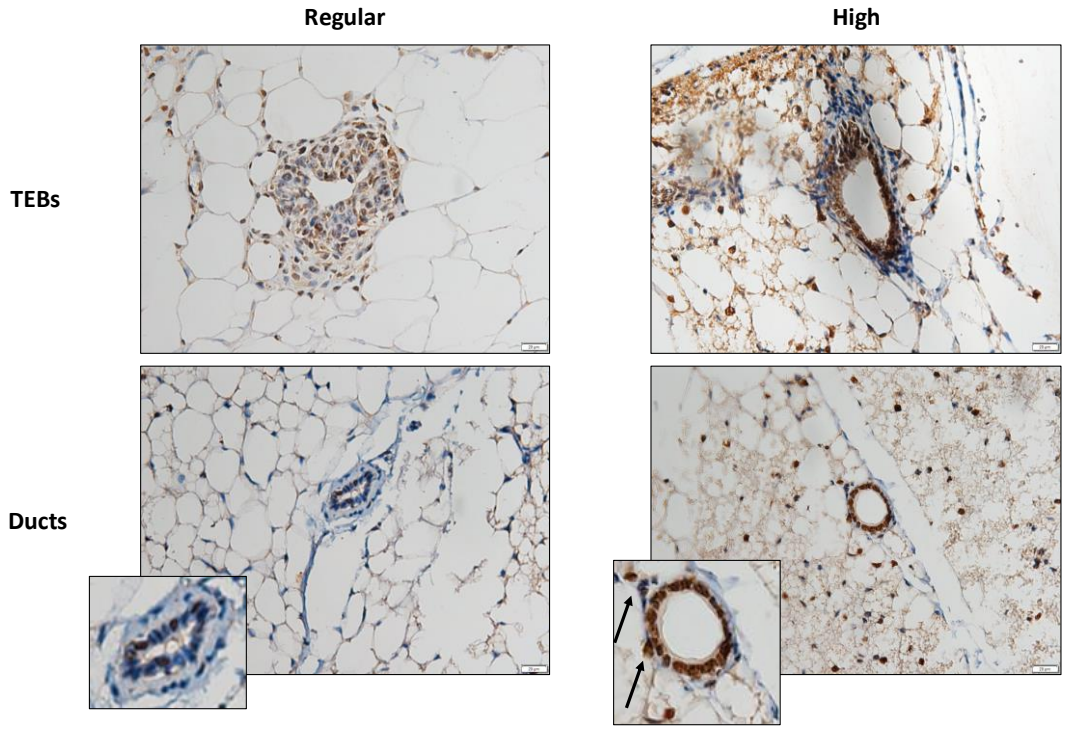


Figure 24. Pubertal Structures RAGE IHC. Representative RAGE IHC stains showing the upregulation of RAGE in adipocytes, epithelial, and stromal cells, believed to be macrophages (arrows) in pubertal structures of mice fed a high age diet, sustaining into adulthood.

Regular diet mice inguinal mammary glands were stained for tissue specific AGEs, showing little to no positive stain in the gland, except for inside the lumen of the ducts. Mice fed a high AGE diet showed increase staining in the epithelial cells of TEBs

and ducts during development, as well as the surrounding stroma. Increased AGE staining was also seen inside the lumen of the structures and the adipocytes of the glands (Figure 25). Low AGE diet showed moderate staining of epithelial, fat and stroma cells similar to high AGE.

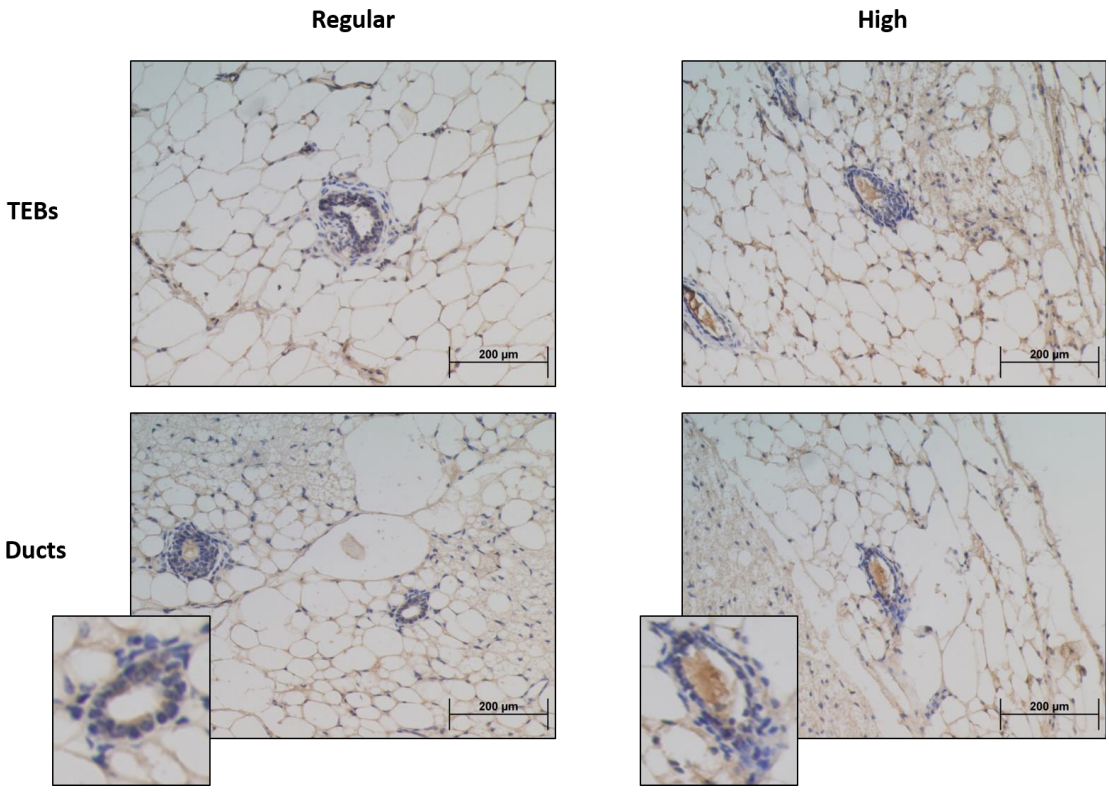


Figure 25. Pubertal Structures AGE IHC. Representative AGE IHC stains, showing presence of AGE in adipocytes, epithelial, and stromal cells in pubertal structures of mice fed a high age diet, sustaining into adulthood.

Discussion and Alternative Approaches

In this aim, we showed that mice fed a diet high in AGEs leads to significant changes in pubertal mammary gland development. Monitoring food consumption and weight gain of the mice fed our experimental and control diets allowed us to limit the variability of obesity or over eating to cause the observed changes. Analysis of our diets

showed increased exogenous AGEs in the cooked high AGE food, low AGE showed moderate AGE content, but still contained the precursors for AGE formation. Evaluation of circulating AGEs in serum and tissue specific AGEs in glands of the mice showed high levels of AGE in mice fed the high AGE diet, as well as moderate levels of AGE in the low AGE diet compared to regular. This led us to believe the low AGE diet was acting as a source of endogenous AGEs as normal metabolism may be leading to the formation of AGEs from the precursors of the diet [1, 2, 4]. This in part explained the trend towards the high AGE diet phenotypic changes observed in the mice fed a low AGE diet. Suggesting the low AGE diet was able to mediate some of the effects observed in the mice fed a high AGE diet, but to in some cases a less significant manner. This was later enforced when we observed increased RAGE expression in the glands of high/low AGE mice compared to regular, showing a possible positive feed-forward loop of RAGE expression via AGE:RAGE signaling [11].

TEBs a characteristic structure of pubertal mammary gland development were significantly affected by a diet high in AGEs, including increased TEB size and number. This may be as a resultant of pro-inflammatory signaling via AGE:RAGE leading to increased activation of fibroblasts and recruitment of macrophages but this remains to be confirmed. Knockout mouse models of mammary gland development have shown these stromal cells to be important in the development and management of TEBs, especially macrophages which when removed lead to a decrease in TEB number and TEB size, opposite of what we observed [11, 28, 29]. Interestingly, increased TEB presence sustained into adulthood, which is a time of quiescence, these structures are similar to terminal ductal lobular unit 1 (TDLU1) known to be the main initiation site of human breast cancer, possibly increasing breast cancer incidence by having these structures still present. TEB size may have also been affected by increased macrophage

recruitment as macrophages are known to secrete factors to promote proliferation, possibly leading to an increase in size [15, 26, 33, 44]. This increased proliferation may explain the thickening of the TEBs and ducts observed in H&E of high AGE mice.

Interestingly, reduction of macrophages in knockout models also resulted in a reduction of ductal branching, where we observed an increase with pubertal high AGE diet, possibly via the increased recruitment of macrophages (which we observed via H&E and later in Specific Aim 2). The main ductal branch-point increased in the high AGE diet inguinal mammary glands was primary branch-points, which are formed from the bifurcation of TEBs, increased TEBs which we observed may be the underlying factor of increased primary and total ductal branching. This increase of TEB bifurcation and primary ductal branching may also explain the delayed ductal extension observed in the high AGE fed mice. Increased bifurcation leads to deflection off the TEBs path to fill the fat pad, this more tortuous path causes an observed delay in extension as the ductal tree will fill the fat pad to fulfill the glands function, but will take longer to achieve. The observed ductal extension delay may also be a cause of change to collagen presence in the ECM as TEBs utilize collagen 1 in the direction of their forward path to fill the gland, activated fibroblasts, macrophage recruitment and direct AGE cross-linking to collagen may affect the path of the TEBs delaying extension [21, 22, 24, 59].

Taken together, this data suggests that a pubertal diet high in AGEs leads to a significant disruption of pubertal mammary gland development, which we believe to be a result of pro-inflammatory signaling via AGE:RAGE signaling leading to increased macrophage and fibroblasts recruitment to pubertal structures of development. These changes to development may lead to effects of breast cancer risk which we further investigate in Specific Aim 2.

A caveat of our experimental design in this specific aim is the non-fasting of animals before collection of blood from cardiac puncture and not properly synching the estrous cycles of the models. Animals may have consumed food before being collected, this may skew the AGE levels of the serum when analyzed showing a false increase of AGE content. Using development models, specifically pubertal models, it is suggested to synch estrous cycles of animals. This synching decreases the variability in the timing of development as one animal could be further into or behind development compared to another. Future directions for this aim would be to carry out serum collection during fasting and synching of animal estrous cycles to decrease any chance of variability in results.

Specific Aim 2: To test the hypothesis that high AGE diet will lead to the formation of pre-neoplastic lesions in adulthood. Pubertal mammary gland development is a tightly regulated process of cellular communication between stromal and epithelial cells. Immune cells such as macrophages play any important role in the development process [28, 29]. AGE:RAGE signaling leads to a pro-oxidant and pro-inflammatory environment, which we see leads to a dysregulation of pubertal mammary gland development. Interestingly, aberrant activation of NF-κB by transgenic mouse model showed that this known downstream factor of AGE:RAGE signaling, led to the formation of DCIS like structures, possibly by increased and sustained immune response via its activation [15]. These observed changes caused by AGEs during pubertal development lead us to determine if the effects of these reactive sugar metabolites may cause the development of pre-neoplastic lesions as result of AGE:RAGE signaling.

- **Task 1:** Evaluate the presence of pre-neoplastic lesions among the diets via H&E:
    1) Quantify the presence of ADH and MIN structures in pubertal and adult glands
- **Task 2:** Evaluate proliferation among the diets via Ki67 IHC:
    1) Perform Ki67 IHC on slices from pubertal and adult glands
    2) Quantify proliferation of ducts, TEBs and pre-neoplastic lesions
- **Task 3:** Evaluate aberrant morphology of TEBs, ductal structures and pre-neoplastic lesions:
    1) Perform IHC with aSMA to assess myoepithelial organization and fibroblast activation in stroma
    2) Perform IHC with F4/80 to assess macrophage recruitment

Experimental Design

H&E. H&E techniques from Specific Aim 1 were utilized to assess the formation of pre-neoplastic lesions of pubertal and adult inguinal mammary glands.

RAGE/AGE IHC. IHC techniques from Specific Aim 1 were utilized to assess changes in RAGE and AGE presence in pre-neoplastic lesions of pubertal and adult inguinal mammary glands.

Ki67/SMA IHC. IHC techniques were used to assess proliferation, myoepithelial structure and fibroblast activation and macrophage presence in the mammary gland. Paraffin embedded inguinal tissue was sliced 5 µm thick and utilized for IHC. Slides were deparaffinized at 60º C for 1 hour. Followed by a series of washes to rehydrate the tissue: 2X xylene for 20 minutes each; air dry for 15 minutes; 3X 100% EtOH, 2X 95% EtOH, 1X 70% EtOH, and 1X 50% EtOH for 10 minutes each; 3X diH_20 for 5 minutes each. For antigen retrieval Vector unmasking solution (Vector Labs, Burlingame, CA) was used at a 1:100 dilution. Slides were placed in a pap jar containing the diluted unmasking solution, brought to boiling and placed in a vegetable steamer containing the diluted solution, for 30 minutes at 90°C. The slides were then placed in a pap jar containing 0.3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ) for 30 minutes to remove any endogenous peroxidase, then washed in 1X phosphate-buffered saline containing 0.01% Tween-20 (PBST) (Fisher Scientific, Fair Lawn, NJ) for 5 minutes. Slides were then incubated in VECTASTAIN® horse serum (Vector Labs, Burlingame, CA) to block non-specific binding for 20 minutes at RT in a humidified chamber. Primary antibody was diluted using 2.5% horse blocking serum diluted with 1X PBS and applied to slides to incubate overnight at 4°C in a humidified chamber (Table 4.). Primary antibody (Thermo Fisher Scientific, Waltham, MA) was then removed by washing 3 times in 1X PBS for 5 minutes on an orbital shaker. Followed by incubation with

VECTASTAIN® secondary antibody solution (Vector Labs, Burlingame, CA) for 30 minutes at RT in a humidified chamber. Slides were washed again 3 times in 1X PBS for 5 minutes on an orbital shaker. Samples were then incubated with SIGMAFAST™ DAB (Sigma-Aldrich Co., St. Louis, MO) to activate the signal, then placed in diH_20 3 times for 5 minutes to stop enzymatic reaction. Hematoxylin (Fisher Scientific, Fair Lawn, NJ) was placed on the tissue and placed into diH_20. Followed by a series of washes to complete the counter stain: HCL-EtOH for 90 seconds; NH_4OH-EtOH for 45 seconds; diH_20 for 3 minutes; Scott's Buffer for 1 minute. Stained slides were then dehydrated by a series of washes: 3-diH_20 for three minutes each; 1X 50% EtOH, 1X 70% EtOH, 2X 95% EtOH, and 3X 100% EtOH for 5 minutes each; air dry for 15 minutes; 2X xylene for 10 minutes each. Glass cover slips were then mounted on top of slides using Permount® (Fisher Scientific, Fair Lawn, NJ). Imaging was completed using an Olympus DP80 microscope (Olympus American, Inc. Center Valley, PA).

Table 4. Antibody Information for Specific Aim 2 IHC Methodology

	Ab Number	Dilution	Secondary Ab	DAB	Hematoxylin
Ki67	RM-9106	1:200	Rabbit	5 min	3 min
SMA	PA5-18292	1:100	Goat	15 sec	1 min

Ki67 Quantification. Images were analyzed using ImageJ software by counting positive or brown stained cells of pubertal structures and pre-neoplastic lesions. Non-positive stained cells were counted and totaled to determine the percent positive of each structure, which were then averaged for each endpoint. Hyperproliferative ducts were ductal structures shown to be over 50% positive (as established by our research team), the presence of these structures was analyzed for each endpoint.

F4/80 IHC. In order to assess macrophage localization to the TEBs, ducts and pre-neoplastic lesions in the developing and adult mammary gland, paraffin embedded inguinal tissue was sliced 5 µm thick and utilized for IHC. Tissue was stained thanks to the Hollings Cancer Center Biorepository and Tissue Analysis shared resource. Primary antibody for F4/80 (14-4801-82, Thermo Fisher Scientific, Waltham, MA) was utilized by the shared resource to complete the stain. Imaging was completed using an Olympus DP80 microscope (Olympus American, Inc. Center Valley, PA).

Results

High AGE and Low AGE diet lead to the formation of pre-neoplastic lesions during late puberty which are, sustained into adulthood. While looking for morphological changes of pubertal structures in H&E stained mammary glands, we observed the formation of potential early stage pre-neoplastic lesions during puberty, as well as late stage in adult mammary glands. This prompted us to look further into the formation of pre-neoplastic lesions during puberty and adulthood of inguinal glands. We observed early stage atypical ductal hyperplasia (ADH) in high AGE and low AGE diet during puberty (Figure 27 & 29), as well as mammary intraepithelial neoplasia (MIN), which sustained into adulthood in both diets (Figure 28 & 29). These structures were structurally similar to DCIS that is seen in human breast cancer; thickening of the epithelial, increased stromal (containing macrophages and activated fibroblasts), hyperproliferation and disorganization of structure caused by disruption of the myoepithelial layer (Figure 26) [31].

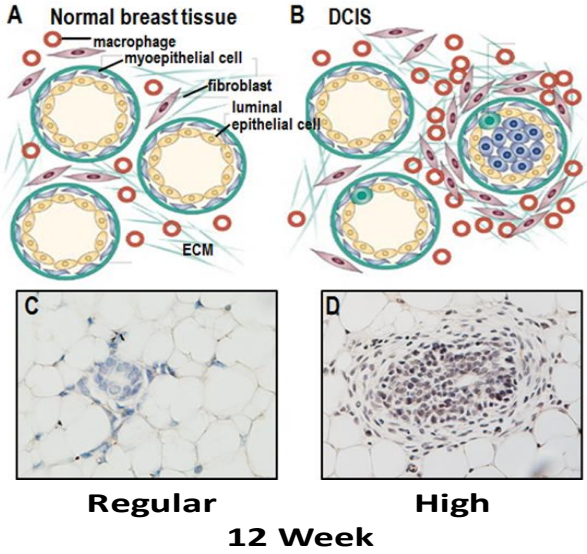


Figure 26. High/Low AGE Diet Leads to Formation of Pre-Neoplastic Lesions. **(A)** In normal breast tissue, stromal cells, including macrophages and fibroblasts are present within the ECM. **(B)** In DCIS, the lumen contains proliferating carcinoma cells and the wall of the duct is irregular. Increased recruitment of fibroblasts and macrophages is observed in the ECM. Figure adapted from (28). Representative image of ducts from 12 week old mice fed a regular **(C)** or high AGE **(D)** diet.

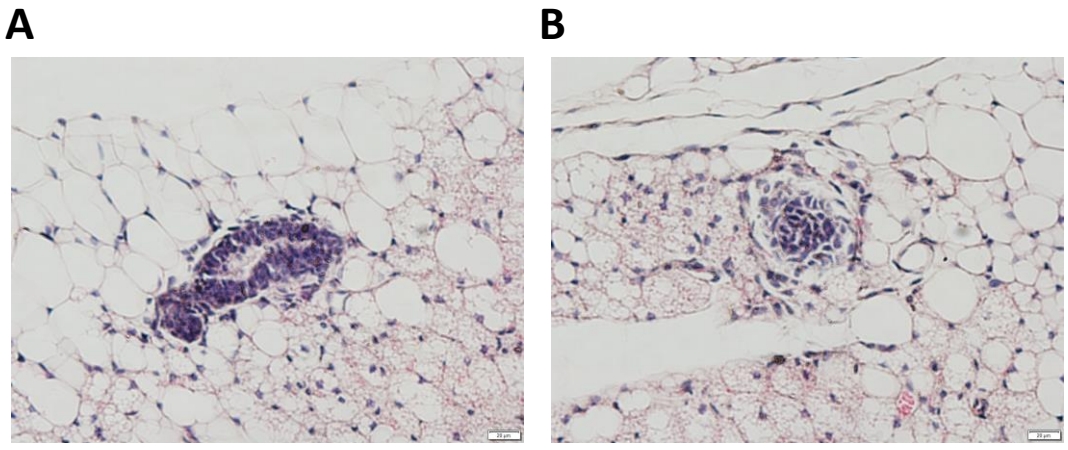


Figure 27. Pubertal Pre-Neoplastic Lesions. Representative images of H&E stained mammary gland tissue sections from 8 week old mice fed a high AGE diet. **(A)** Atypical epithelia structures representing early stage, low grade atypical ductal hyperplasia (ADH). **(B)** Atypical lesions progress to high grade mammary intraepithelial neoplasia (MIN). These larger intraepithelial neoplastic lesions fill the ductal lumen but do not invade the basement membrane. These lesions appear similar to human DCIS.

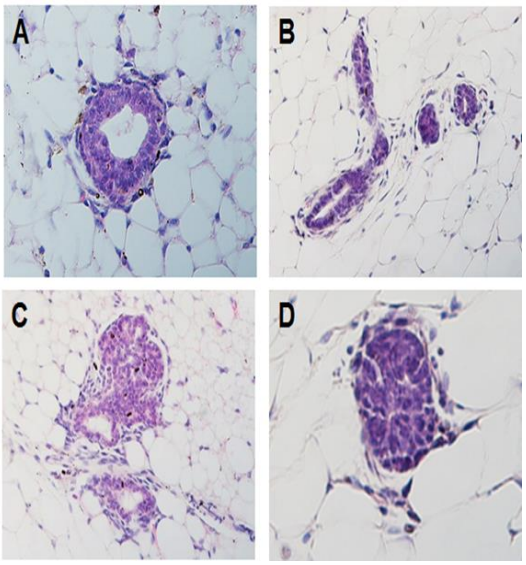


Figure 28. Adulthood Pre-Neoplastic Lesions. Representative images of H&E stained mammary gland tissue sections from wild type mice fed a high AGE diet. **(A & B)** Atypical epithelia structures representing early stage, low grade ADH. **(C & D)** Atypical lesions progress to high grade MIN. These larger intraepithelial neoplastic lesions fill the ductal lumen but do not invade the basement membrane. These lesions appear similar to human DCIS.

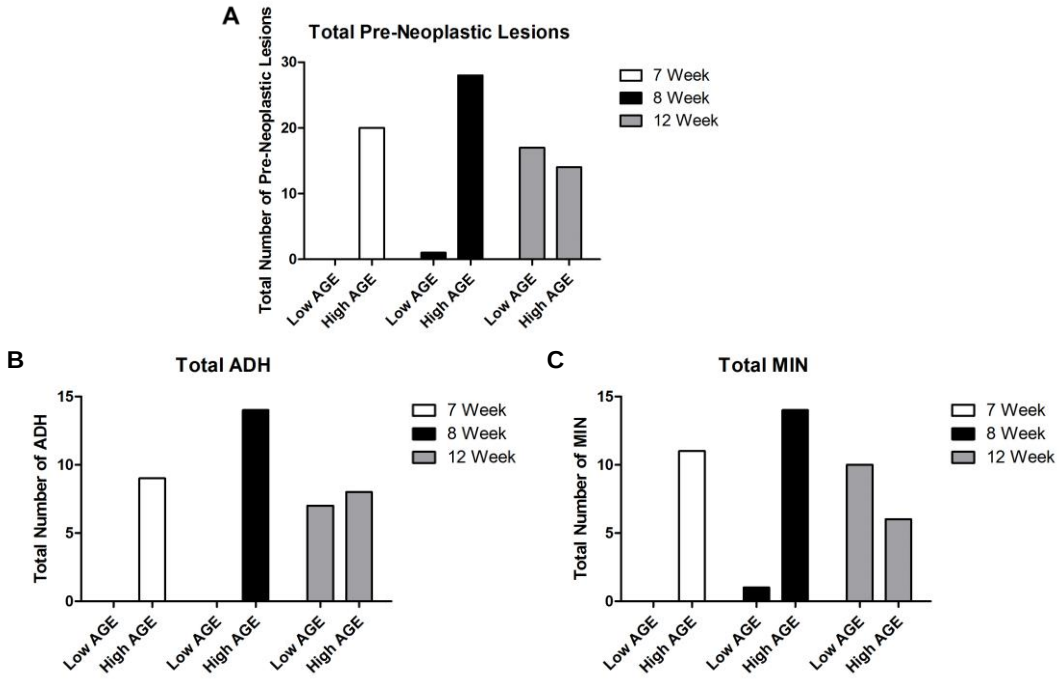


Figure 29. Amount of Pre-Neoplastic Lesions. Total number of pre-neoplastic lesions during late puberty and adulthood in the inguinal mammary glands of mice fed a high and low AGE diet **(A)**, further broken down into ADH **(B)** and MIN **(C)**.

Pre-neoplastic lesions showed increased presence of RAGE and AGE. As stated previously, AGE:RAGE signaling leads to a positive feedback loop leading to increased RAGE expression prompting us to assess changes in RAGE and tissue specific AGE presence of pre-neoplastic structures via IHC analysis using inguinal glands. The pre-neoplastic lesions observed in the high/low AGE diets at puberty and adulthood showed increased RAGE expression in the epithelial cells and stromal cells, as well as the surrounding adipocytes (Figure 30). The pre-neoplastic lesions were also shown to have positive AGE staining in the epithelial and stromal cells of pubertal and adult mice glands (Figure 31).

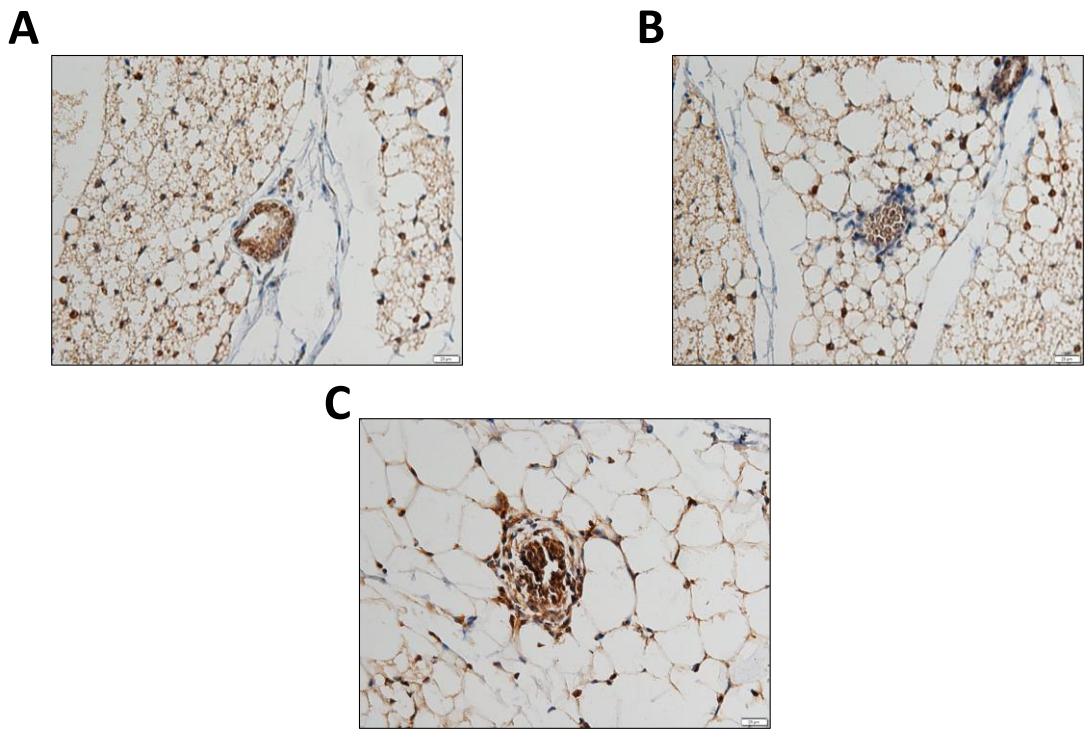


Figure 30. Pre-Neoplastic RAGE IHC. Representative RAGE IHC stains, showing increased presence of RAGE in pre-neoplastic lesions in ADH **(A)** and MIN **(B)** during puberty and MIN **(C)** in adulthood of mice feed a high AGE diet.

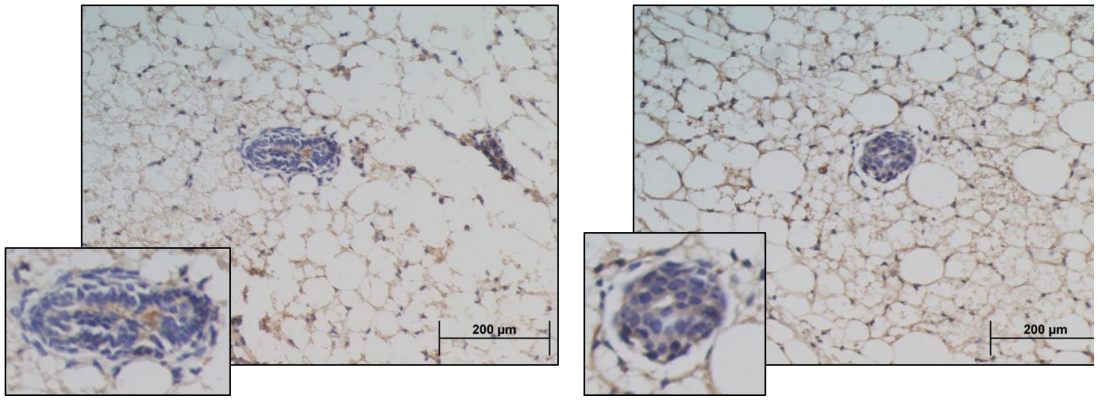


Figure 31. Pre-Neoplastic Lesions AGE IHC. Representative AGE IHC stains, showing increased presence of AGE in pre-neoplastic lesions of high AGE **(A)** and low AGE **(B)** adult glands.

High AGE diet results in increased proliferation in TEBs and ducts during puberty, as well as pre-neoplastic lesions. Increased TEB size, morphological changes and the formation of pre-neoplastic lesions prompted us to assess the changes in proliferation of pubertal structures and pre-neoplastic lesions during pubertal development and adulthood among the diets via Ki67 IHC analysis of inguinal glands. Using Ki67 to assess cellular proliferation during puberty, a diet high in AGEs lead to increased proliferation of TEBs and ducts during pubertal time points (Figure 32). The diet also lead to increased presence of hyper-proliferative ducts in the developing glands, or ducts (defined as being over 50% positive for Ki67 staining) (Table 5). It was observed that high AGE diet lead to formation of ADH and MIN structures, these structures were highly present in high AGE compared to both low AGE and regular diet, which contained no such structures (Figure 29). Proliferation was assessed for each structure and showed them to be highly proliferative, even into adult hood, normally a time of quiescence (Figure 33).

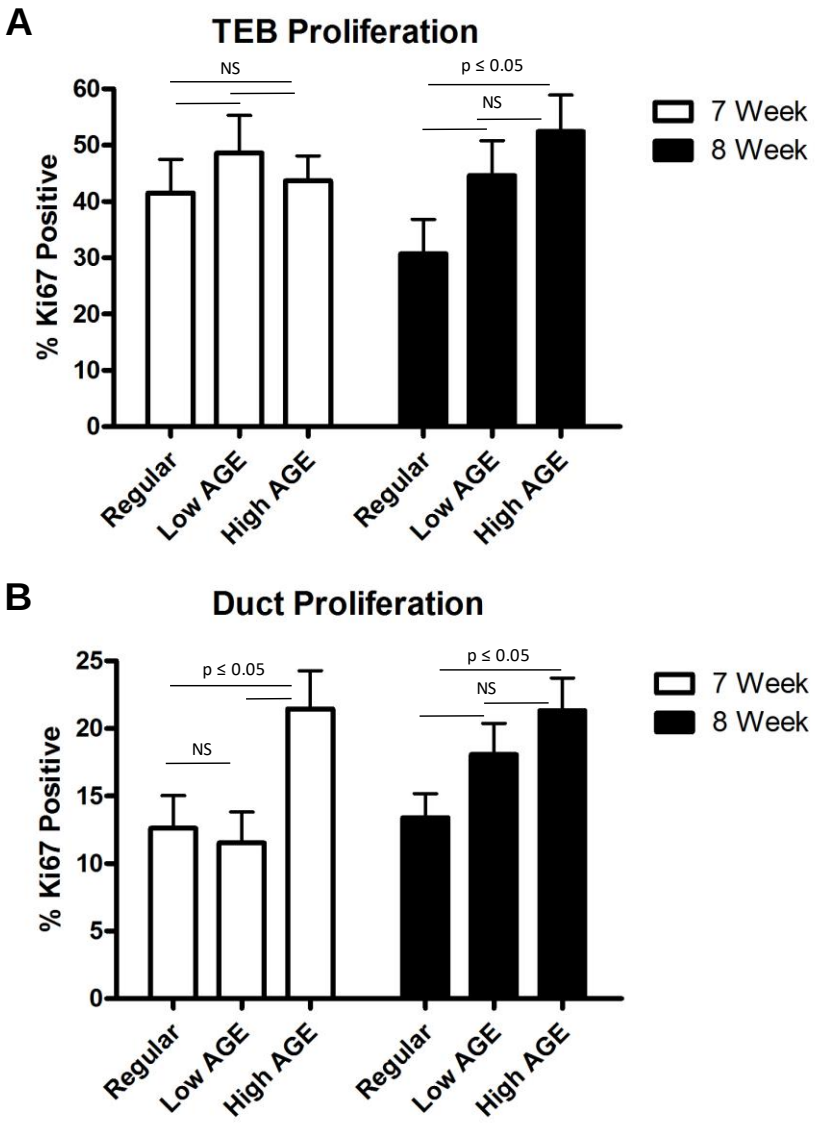


Figure 32. TEB and Duct Proliferation. Ki67 IHC showed increased proliferation of TEBs **(A)** and ducts **(B)** during late puberty in mice fed a diet high in AGEs.

Table 5. Percentage of Glands Containing Hyperproliferative Ducts			
	Regular	**Low AGE**	**High AGE**
7 Week	25%	25%	60%
8 Week	0%	33%	100%

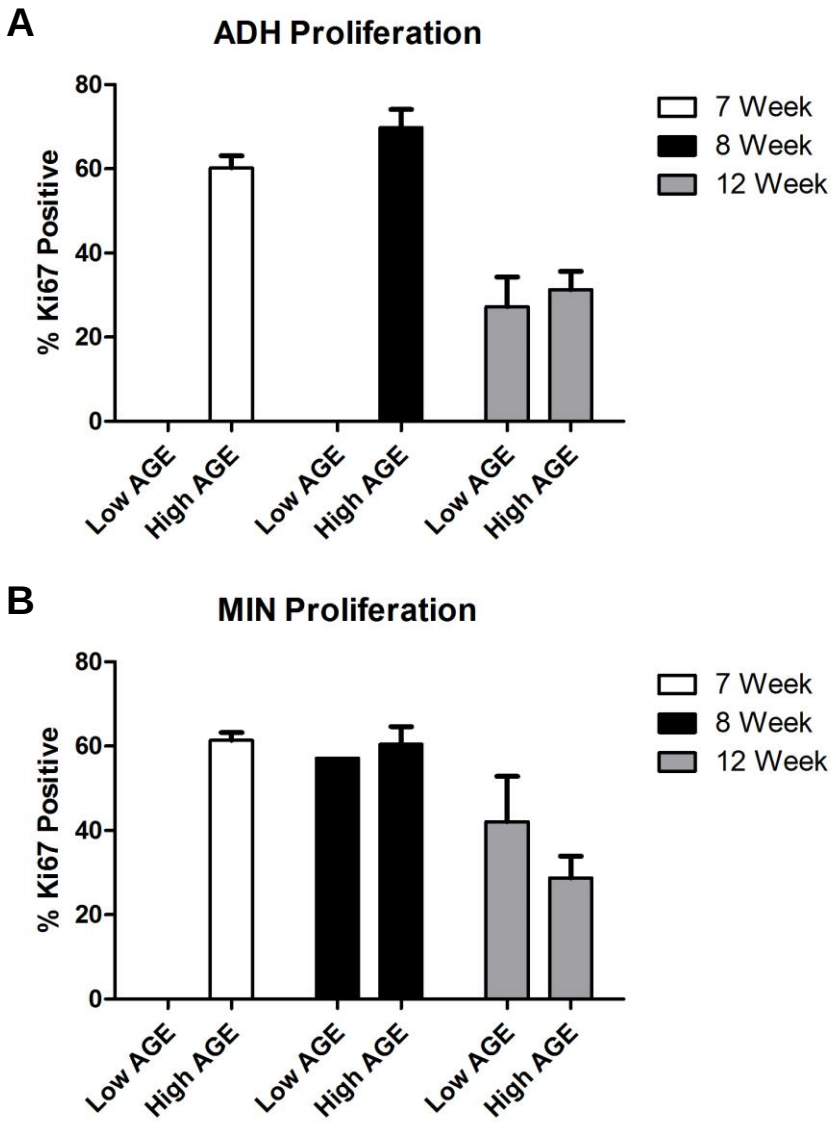


Figure 33. Pre-Neoplastic Lesion Proliferation. Ki67 IHC showed proliferation of ADH **(A)** and MIN **(B)** structures during late puberty in mice fed a high AGE and low AGE diet.

High AGE diet results in the disruption of the myoepithelial layer in developing structures as well as pre-neoplastic lesions. We also observed disruption of structure and migration of cells into the fat pad, prompting us to examine the myoepithelial layer via SMA IHC of inguinal glands. High AGE diet resulted in disruption of the myoepithelial layer in TEBs and ducts of the developing glands (Figure 34). When looking at ADH and MIN structures in the pubertal and adult glands fed the experimental diets, disruption of the myoepithelial layer was observed in these pre-neoplastic lesions, (Figure 35) a common characteristic of human DCIS [31, 43].

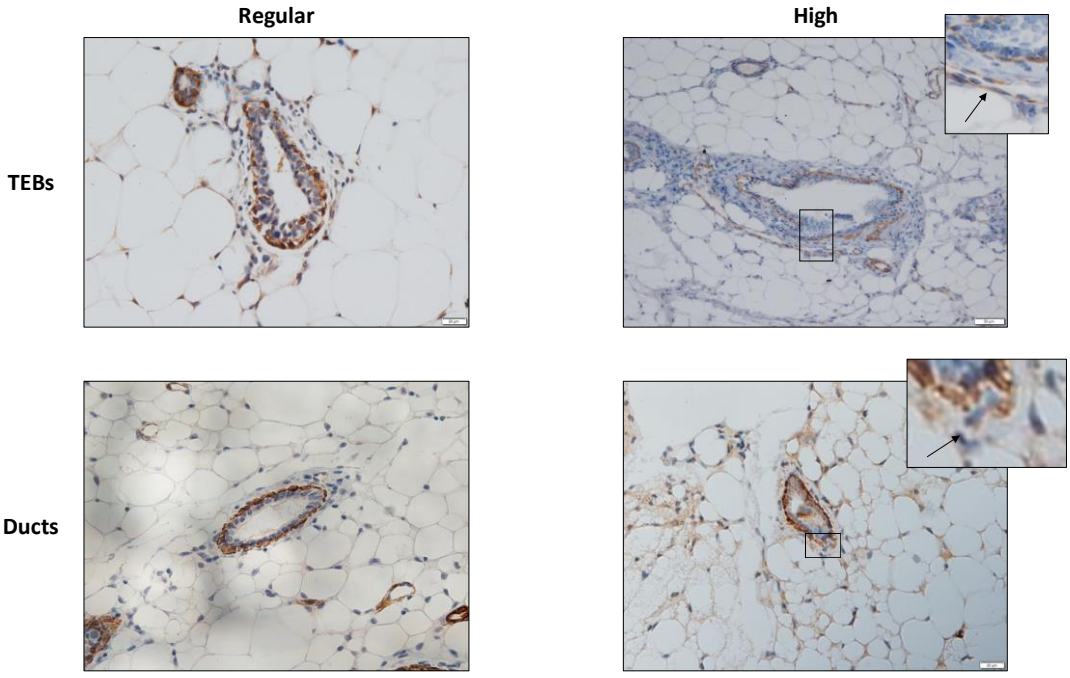


Figure 34. Pubertal Structures SMA IHC. Representative image of SMA IHC stained mammary gland tissue sections from wild type mice fed a regular and high AGE diet. High AGE diet showed disruption of the myoepithelial layer in TEBs and ducts of the developing pubertal glands. High AGE diet also showed increased activated fibroblasts surrounding the TEBs and ducts of the developing pubertal glands (arrows).

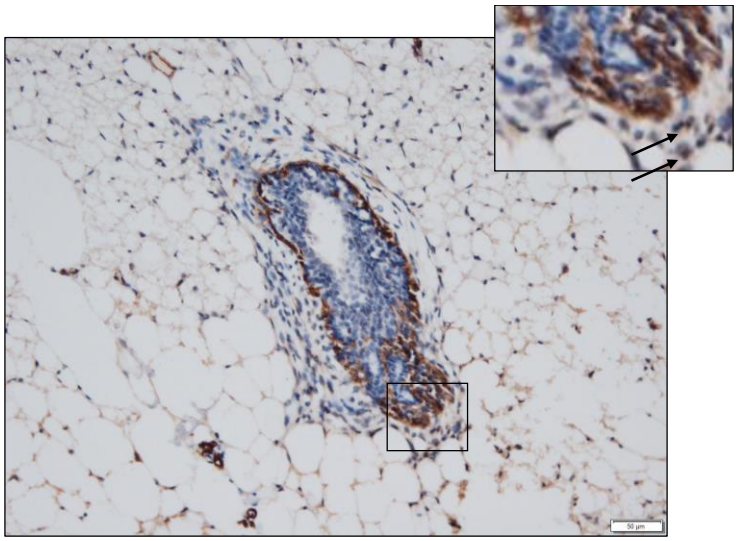


Figure 35. Pre-Neoplastic Lesions SMA IHC. Representative image of SMA IHC stained mammary gland tissue sections from wild type mice fed a high AGE diet. High AGE diet showed disruption of the myoepithelial layer in pre-neoplastic lesions of the pubertal and adult glands. High AGE diet also showed increased activated fibroblasts surrounding pre-neoplastic lesions of the adult glands (arrows).

High AGE diet results in the increased activation of fibroblasts and macrophage recruitment surrounding developing structures and pre-neoplastic lesions. Morphological analysis showed increased fibroblasts presence around pubertal structures and pre-neoplastic lesions. SMA is a common marker of fibroblasts activation, while staining with SMA for disruptions in the myoepithelial layer, it was observed that the high AGE diet resulted in an increase in activated fibroblasts recruited to developing structures during puberty (Figure 34 insert). In adulthood, high AGE diet showed to increase activated fibroblast recruitment surrounding the pre-neoplastic lesions observed (Figure 35 insert).

Morphological analysis also showed that there was increased recruitment of macrophages to developing structures and pre-neoplastic lesions of the gland. AGE:RAGE signaling is known to lead to a pro-inflammatory response, and immune cells are known to play an important role in development, pushing us to further look at

macrophage presence by F4/80 IHC. High AGE diet resulted in increased macrophage recruitment around developing structures during puberty compared to control diet (Figure 36 i & ii). The increased recruitment sustained into adulthood and showed to be prevalent around and inside pre-neoplastic lesions of the mice fed high AGE (Figure 36 iii). Increased macrophage recruitment and activated fibroblasts is a common characteristic of DCIS [31, 43].

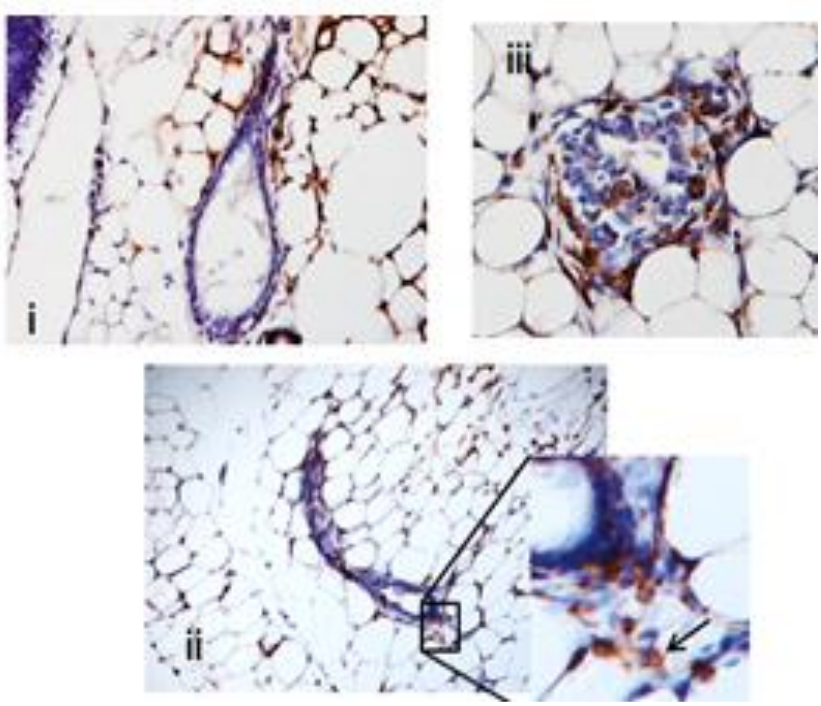


Figure 36. Increased Macrophage Recruitment. Representative images of F4/80 IHC stained mammary gland tissue sections or regular fed mice (**i**) from wild type mice fed a high AGE diet (**ii**). High AGE diet showed increased macrophage recruitment surrounding pre-neoplastic lesions of the adult glands (**iii**) as well as TEBs during development (**ii**).

Discussion and Alternative Approaches

During late pubertal development, it was observed histologically that low and high AGE diets were resulting in the formation of ADH and MIN structures, which sustained into adulthood. These pre-neoplastic lesions are structurally similar to

formation and structure of human DCIS [31]. These structures stained positive for tissue specific AGEs and RAGE, possibly from the upregulation of RAGE caused by AGE:RAGE signaling [4, 11, 16]. Increased macrophage recruitment to the gland was observed in F4/80 stained glands surrounding developing TEBs and ducts in high AGE mice. The increased macrophage recruitment observed may contribute to development changes, such as increase TEB number/size and ductal branching seen in Specific Aim 1. High AGE adult mammary glands stained for F4/80 also revealed increased macrophage presence and infiltration in the pre-neoplastic lesions, a common characteristic of human DCIS [26, 44, 45].

Proliferation was assessed in the developing mammary glands possibly from AGE:RAGE signaling leading to pro-proliferation factors being released from macrophages, leading to observed phenotypic changes [10, 11, 13, 26, 44, 45]. It was observed that a diet high in AGEs led to increased proliferation of TEBs and ducts during late puberty as well as increased hyperproliferative duct presence throughout development. This increased proliferation was sustained into adulthood as the pre-neoplastic lesions showed to be proliferative throughout development, a characteristic of DCIS [31, 56, 60]. However, we did not assess apoptosis of these structures to determine cellular turnover, a future direction we plan to assess using IHC markers of apoptosis (e.g. apoptosis inducing factor (AIF), BAX and Caspase-1)

Structure of the myoepithelial layer was observed via SMA staining to assess if the diet high in AGEs was disrupting the layer supporting a more invasive phenotype. During pubertal development, it was observed that a pubertal diet high in AGEs led to a disruption of the myoepithelial layer in TEBs and ducts, this disruption carried into adulthood as the pre-neoplastic lesions in the adult glands showed to have a disruption in the myoepithelial cells. This disruption may establish a pre-disposition to aggressive

cancer as disruption of the layer is associated with IDC [31, 43, 56]. This disruption may result from protein and DNA changes via the pro-oxidant and inflammatory signaling of AGE:RAGE, and possible effects sustained to the cap cells of the TEBs or mammary stem cells known to differentiate into myoepithelial cells [24, 59, 61].

Activated myofibroblasts expressing the marker SMA were also stained while looking at myoepithelial structure, the SMA marker is commonly associated with CAFs [47]. We observed that high AGE fed mice showed increased activated fibroblasts in the stroma of TEBs during development. These activated fibroblasts may also lead to the changes we observed in development in Specific Aim 1 as fibroblasts are known to secrete factors, such as growth factors, proteases and cytokines aiding in the development of the gland as well as recruiting immune cells, such as macrophages. Pre-neoplastic lesions during puberty and into adulthood of high AGE fed mice showed recruitment of activated fibroblasts to the stroma, a common characteristic of human DCIS [31, 45-47].

Taken together, this data suggests that a pubertal diet high in AGEs leads to a recruitment of macrophages and activated fibroblasts to developing structures of pubertal mammary glands and increased proliferation, possibly leading to the increased TEB number, size and branching as well as the delayed extension we observed in Specific Aim 1. A diet high in AGEs also led to the development of pre-neoplastic lesions in late puberty which further progressed in adulthood. These lesions shared similar characteristics to human DCIS: increased macrophage presence, increased fibroblast recruitment and activation, increased proliferation and disruption of the myoepithelial layer [31, 45-47]. These changes to development and formation of pre-neoplastic lesions may lead to increased breast cancer risk with exposure to oncogenic insult, as seen in diets such as high fat and high n-6 PUFA [38, 57].

A caveat with the experimental design in this specific aim is the staining for macrophages using an F4/80 antibody, which has also been shown to stain eosinophils. Eosinophils also play an important role in mammary gland development and possible positive staining of macrophages could be eosinophils [28, 29]. However, distinct morphological differences help to distinguish between the two cell types. A future direction for this aim would be to use a dual stain method to better distinguish between macrophage and eosinophil recruitment to structures of the glands using immunofluorescence with specific macrophage markers (CD68) and eosinophil markers (Siglec-F). Quantification of any IHC stain is imperfect and correlation with positive staining and observed changes does not imply a positive connection between the two. Even though we see increased macrophage and activated fibroblasts recruitment in mice fed the high AGE diet, it is important to investigate the AGE:RAGE signaling pathway further to determine by which AGEs and RAGE are inducing the changes to development and formation of the pre-neoplastic lesions. We look to investigate this association utilizing cell culture and transgenic mouse models, further discussed in the future directions.

Future Experiments

Our collective data show a link between consumption of a high AGE diet with significant disruption of normal pubertal mammary gland development, possibly leading to increased cancer risk via the formation of pre-neoplastic lesions. However, the exact mechanism of these drastic changes is not known and something we look to investigate further as well as cancer incidence among the diets (Figure 37).

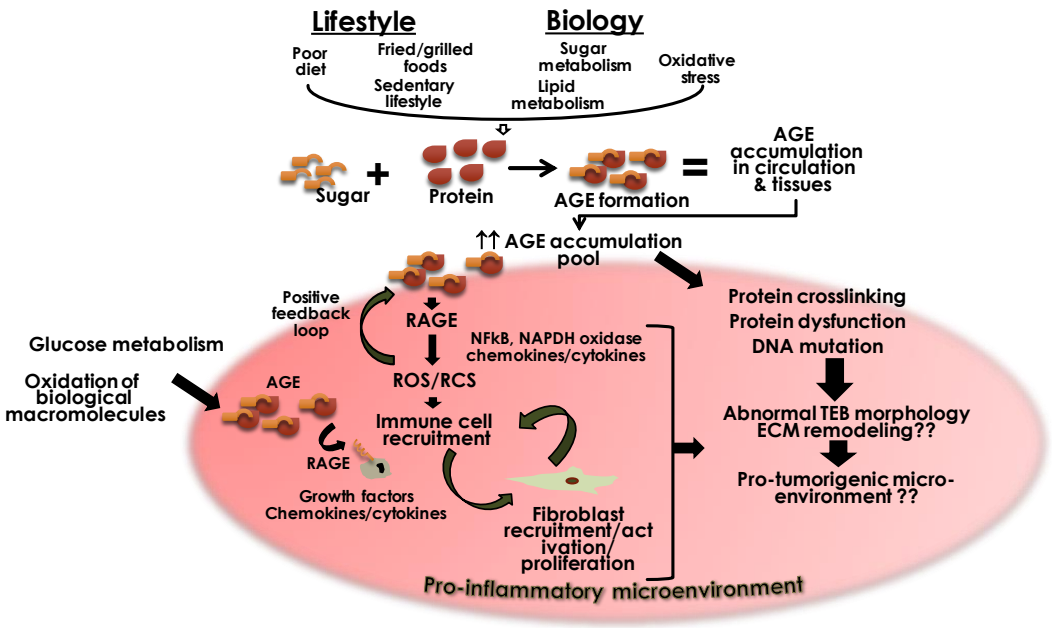


Figure 37. Working Hypothesis. Our current working hypothesis of AGE:RAGE signaling, leading to the observed phenotypic changes and pre-neoplastic lesions observed.

Macrophage and Fibroblast Recruitment in High AGE Diet. We first look to further establish the recruitment of immune cells and activation of fibroblast to the stroma of surrounding developing structures in mice fed a high AGE diet. We plan to continue with our normal development model of FVB/n female mice beginning their diets at 3 weeks of age, this time feeding them for a period of 4 weeks (7 weeks of age).

Using co-immunofluorescent staining using markers for stromal cells: macrophages (F4/80 and CD68) and fibroblasts (vimentin and FSP1) as well as cells for TEB identification: basal myoepithelial cells (αSMA), cap cells (p63) and body cells (E-cadherin) we look to confirm the recruitment of these stromal cells to TEB structures, as well as the cell type that has AGE mediated upregulation of RAGE via co-immunofluorescent staining using fibroblast and macrophage markers with RAGE. Using our 7-week-old mice endpoint we look to define the AGE/RAGE association with the activation of macrophages using flow cytometry by co-expression of CD11b/CD68 or pro-inflammatory ("M1') phenotype or tumorigenic ("M2") from expression of CD86/iNOS or CD206/Arginase1 [62]. AGE/RAGE will also be assessed to determine association with macrophages. We expect to see increased mis-localized recruitment of macrophages to developing structures with a switch to a pro-tumorigenic phenotype in high AGE diet compare to regular, AGE/RAGE staining is expected to be seen co-localized to macrophages in the high AGE diet fed mice. Some effects of low AGE diet are expected as they represent an endogenous source of AGE, however the effects are expected to be less severe and less prominent in the glands.

Fibroblasts Activation in the High AGE Diet. We then plan to further understand the AGE/RAGE association with activation and recruitment of fibroblasts to TEBS during puberty. Fibroblasts from 7-week-old mice will be identified via common markers (FSP/vimentin) and distinctive spindling morphology, we will isolate from contaminating stromal and epithelial cell population to establish cell culture. Using flow cytometry, western blot and qPCR, activation of fibroblasts will be assessed with FAP and COL1A1 expression as they are specific markers of fibroblasts and are upregulated in activated fibroblasts [63]. We expect to see increased mis-localized recruitment of activated fibroblasts (high FAP/COL1A1, low CD36) to TEBs with a switch to a pro-

tumorigenic microenvironment in high AGE diet compare to regular. AGE/RAGE staining is expected to be co-localized to fibroblasts in the high AGE diet. Effects of low AGE diet are expected to be seen (endogenous AGE), however the effects are expected to be less severe and less prominent in the glands.

Identification of AGE Species in the Mammary Gland. Depending on the manner of AGE formation whether it be formed through glycation or oxidation, etc. multiple species of AGEs are possible depending on the proteins that are being glycated. Variations in the formation of AGEs can vary the pathogenic affect as well as the effect on pubertal mammary gland development. Using dot blot, western blot and fluorescent cytochemistry, we will look at various species of AGEs using specific antibodies to identify potential pathways and mechanism. As AGE formation, can result from various mechanisms, such as lipid peroxidation, glucose oxidation, and the Maillard reaction, to list a few [1, 2]. We expect to see species-specific AGE presence and localization to surrounding epithelial cells or macrophages/fibroblasts providing information on the mechanism leading to the change in development and possible pro-tumorigenic environment.

Determine Timing of Phenotypic Change in High AGE Diet. Another direction would to be to determine the timing of abnormal development and stromal recruitment during pubertal development, using our same model we would collect mice after exposure to the diets for 1, 2 and 3 weeks. The mice will be analyzed using similar analysis used in the thesis, such as: ductal extension, ductal branching, TEB number and size, as well as morphological and stromal changes with H&E. We anticipate that changes will occur rather quickly, around the 1 week timepoint with increased macrophage recruitment followed by increased fibroblast recruitment as seen in our hypothesized mechanism (Figure 37).

Determine the Role of AGE:RAGE Signaling in Observed Phenotypic Changes. To determine the mechanism behind our observed changes in development we look to verify if the dysregulation is caused from signaling through the AGE:RAGE pathway. FVB/n RAGE -/- mice will be fed the diets from weaning (3 weeks) to the 7-week timepoint, at which point the glands will be assessed as described previously comparing all data to FVB/n wild type (RAGE +/+) mice fed the same diets. RAGE -/- mice fed a regular diet will be compared to RAGE +/+ mice fed a regular diet as a control to see the effects of RAGE loss alone on mammary gland development.

Using co-culture models of non-transformed mammary epithelial cells (HC11-Mouse and MCF10A-Human) and isolated fibroblasts/macrophages of RAGE -/- and RAGE +/+ mice, we can further assess the need for RAGE in the activation of macrophages and fibroblasts. Monitoring the expression/activation levels of various signaling pathways such as MAPKs, ERK and the positive feedback loop of RAGE itself can further help us understand the mechanism leading the heightened inflammatory response (Figure 36). During activation, macrophages and fibroblasts secrete specific factors to their function in the developing gland (MMPs, IL-6, TGFβ, FGF2 and PDGF), media from macrophages and fibroblasts will be collected to assess the secreted factor profile, as well as presence of receptors to assess changes under the influence of AGE and RAGE causing a pro-inflammatory and oxidant environment. We anticipate a significant proportion of activated fibroblast and macrophages in a pro-tumorigenic state, expressing a specific cytokine/chemokine profile. We expect to see an increase in CSF1 and CSF1R expression as it is the main receptor and ligand expressed in recruitment of macrophages to the developing mammary gland, which we believe will increase from the pro-inflammatory signaling of AGE:RAGE. We expect to see an increase in IL-6 a key regulator of macrophage and fibroblast recruitment/activation [26-29, 33, 34].

We also plan to target RAGE biologically via shRNA and neutralizing antibodies as well as pharmacologically (RAGE Antagonist, FPS-ZM1, Calbiochem). This will allow us to modulate RAGE in specific cell types before administration of AGEs to assess the downstream effects on macrophage and fibroblasts activation. To assess downstream signaling pathways, responsive to AGE/RAGE activation, biological and pharmacological inhibitors specific for NFκB, AKT and ERK (known effectors of the AGE/RAGE signaling axis) will be utilized in the epithelial cells to assess impact of AGE-mediated activation of fibroblasts and macrophages. The effects observed on the RAGE -/- and RAGE +/+ models on downstream components such as: NFκB, AKT and ERK will add insight into the mechanism of AGE induced dysregulation during normal pubertal mammary gland development.

Determine the Impact of Pubertal AGE Ingestion on Breast Cancer Incidence. We look to utilize the C3(tetO-TAg) inducible mouse model of cancer (017719; Jackson Labs, Bar Harbor, Maine) to assess if feeding a high AGE diet creates a pro-tumorigenic microenvironment leading to increased incidence and/or progression in response to an additional oncogenic insult. The C3(tetO-TAg) mice will be bred with MTB (MMTV-rtTA) mice, which will allow doxycycline (dox)-inducible expression of C3-TAg specifically in the mammary epithelium. Transgenic C3(tetO-TAg) and bigenic C3(tetO-TAg)/MTB mice will be fed regular, low AGE and high AGE diets from weaning till maturity (12 weeks of age), at which point they will be given dox water to induce the transgene. Mice will be sacrificed at 4, 8, 12 and 16 weeks post-dox induction to assess tumor initiation and progression. We anticipate that the high AGE diet will lead to accelerated tumor growth and progression, as our data has shown that the high AGE diet creates a pro-tumorigenic microenvironment primed for oncogenic insult.

Assess the Requirement of RAGE in AGE-Mediated Cancer Progression. The C3(tetO-TAg/MTB bigenic mice will be bred to the RAGE -/- mice to create trigenic C3(tetO-TAg)/MTB/RAGE-/- and bigenic C3(tetO-TAg)/RAGE-/- control mice, all maintained on an FVB/n background. As described above, the mice will be fed the regular low and high AGE diets from weaning to adulthood, mice will be sacrificed at 12 weeks to assess the role of RAGE in the formation of pre-cancerous lesions, and the remaining mice will be exposed to dox to induce the transgene. Mice will be analyzed in a similar fashion as described to determine the role of RAGE in cancer progression in the presence and absence of AGE. Based on published studies, we anticipate that RAGE is required for the development of pre-cancerous lesions, with absence of RAGE less severe effects of AGE-mediated tumor progression will be observed.

Significance of Study

Previous studies have looked on the effects of diet, such as high-fat diet on pubertal mammary gland development with relationship to breast cancer progression in adulthood. However, AGEs have been greatly overlooked despite their increased presence in our modern day “Western” diet, sedentary lifestyle and increased consumption of cheap processed foods. We are the first group to establish this link of increased AGE consumption and dysregulation in pubertal mammary gland. Puberty is a time of tight regulation, a time where many adolescents are not necessarily thinking towards the future of their health and cancer risk.

This lack of knowledge may lead to complications of development over time, dysregulations of similar processes that are seen to contribute to tumorigenesis, a consequence we observed in our study. The disruption of development led to structures that are similar to cancerous lesions in human breast. Showing that consumption of a high AGE diet during puberty may lead to predisposition of breast cancer and increased

cancer progression when exposed to oncogenic insult. This groundbreaking insight into the effects of AGE on development and breast cancer risk, enforces the importance of teaching the public, especially adolescents the importance of healthy eating and an active lifestyle.

References

1. Goldberg, T.e.a., *Advanced glycoxidation end products in commonly consumed foods.* Journal of the American Dietetic Association. **104**: p. 1287-1291.
2. Luevano-Contreras, C. and K. Chapman-Novakofski, *Dietary Advanced Glycation End Products and Aging.* Nutrients, 2010. **2**(12): p. 1247-1265.
3. O'Brien, J., P.A. Morrissey, and J.M. Ames, *Nutritional and toxicological aspects of the Maillard browning reaction in foods.* Critical Reviews in Food Science and Nutrition, 1989. **28**(3): p. 211-248.
4. Poulsen, M.W., et al., *Advanced glycation endproducts in food and their effects on health.* Food and Chemical Toxicology, 2013. **60**: p. 10-37.
5. Uribarri, J., et al., *Advanced Glycation End Products in Foods and a Practical Guide to Their Reduction in the Diet.* J Am Diet Assoc, 2010. **110**(6): p. 911-16.e12.
6. Sharma, C., et al., *Advanced glycation End-products (AGEs): an emerging concern for processed food industries.* Journal of Food Science and Technology, 2015. **52**(12): p. 7561-7576.
7. Yamamoto, Y. and H. Yamamoto, *RAGE-Mediated Inflammation, Type 2 Diabetes, and Diabetic Vascular Complication.* Frontiers in Endocrinology, 2013. **4**: p. 105.
8. Delgado-Andradea, C., *Carboxymethyl-lysine: thirty years of investigation in the field of AGE formation* Food and Function, 2016(1).
9. Turner, D.P., *Advanced glycation end-products: a biological consequence of lifestyle contributing to cancer disparity.* Cancer research, 2015. **75**(10): p. 1925-1929.
10. Jin, X., et al., *Advanced Glycation End Products Enhance Macrophages Polarization into M1 Phenotype through Activating RAGE/NF-κB Pathway.* BioMed Research International, 2015. **2015**: p. 732450.
11. Ott, C., et al., *Role of advanced glycation end products in cellular signaling().* Redox Biology, 2014. **2**: p. 411-429.
12. Song, F., et al., *RAGE Regulates the Metabolic and Inflammatory Response to High-Fat Feeding in Mice.* Diabetes, 2014. **63**(6): p. 1948-1965.
13. Younessi, P. and A. Yoonessi, *Advanced Glycation End-Products and Their Receptor-Mediated Roles: Inflammation and Oxidative Stress.* Iranian Journal of Medical Sciences, 2011. **36**(3): p. 154-166.
14. Angeloni, C., L. Zambonin, and S. Hrelia, *Role of Methylglyoxal in Alzheimer's Disease.* BioMed Research International, 2014. **2014**: p. 238485.
15. Barham, W., et al., *Aberrant activation of NF-κB signaling in mammary epithelium leads to abnormal growth and ductal carcinoma in situ.* BMC Cancer, 2015. **15**: p. 647.
16. Byun, K., et al., *Advanced glycation end-products produced systemically and by macrophages: A common contributor to inflammation and degenerative diseases.* Pharmacology & Therapeutics.
17. Serban, A.I., et al., *RAGE and TGF-β1 Cross-Talk Regulate Extracellular Matrix Turnover and Cytokine Synthesis in AGEs Exposed Fibroblast Cells.* PLoS ONE, 2016. **11**(3): p. e0152376.
18. Tago, K., et al., *Receptor for advanced glycation endproducts signaling cascades are activated in pancreatic fibroblasts, but not in the INS1E insulinoma cell line: Are mesenchymal cells major players in chronic inflammation?* Islets, 2016. **8**(5): p. 135-144.

19. Inman, J.L., et al., *Mammary gland development: cell fate specification, stem cells and the microenvironment.* Development, 2015. **142**(6): p. 1028.
20. Macias, H. and L. Hinck, *Mammary Gland Development.* Wiley interdisciplinary reviews. Developmental biology, 2012. **1**(4): p. 533-557.
21. Sternlicht, M.D., *Key stages in mammary gland development: The cues that regulate ductal branching morphogenesis.* Breast Cancer Research, 2006. **8**(1): p. 201-201.
22. Sternlicht, M.D., et al., *Hormonal and local control of mammary branching morphogenesis.* Differentiation; research in biological diversity, 2006. **74**(7): p. 365-381.
23. Butner, J.D., et al., *A hybrid agent-based model of the developing mammary terminal end bud.* Journal of Theoretical Biology, 2016. **407**: p. 259-270.
24. Paine, I.S. and M.T. Lewis, *The Terminal End Bud: the Little Engine that Could.* Journal of Mammary Gland Biology and Neoplasia, 2017: p. 1-16.
25. SM, B., *The development of the terminal end bud in the prepubertal-pubertal mouse mammary gland.* Anat Rec., 1998. **250**(4): p. 459-64.
26. Brady, N.J., P. Chuntova, and K.L. Schwertfeger, *Macrophages: Regulators of the Inflammatory Microenvironment during Mammary Gland Development and Breast Cancer.* Mediators of Inflammation, 2016. **2016**: p. 4549676.
27. Coussens, L.M. and J.W. Pollard, *Leukocytes in Mammary Development and Cancer.* Cold Spring Harbor Perspectives in Biology, 2011. **3**(3): p. a003285.
28. Gouon-Evans, V., E.Y. Lin, and J.W. Pollard, *Requirement of macrophages and eosinophils and their cytokines/chemokines for mammary gland development.* Breast Cancer Research : BCR, 2002. **4**(4): p. 155-164.
29. Gouon-Evans, V., M.E. Rothenberg, and J.W. Pollard, *Postnatal mammary gland development requires macrophages and eosinophils.* Development, 2000. **127**(11): p. 2269.
30. O'Brien, J., et al., *Alternatively Activated Macrophages and Collagen Remodeling Characterize the Postpartum Involuting Mammary Gland across Species.* The American Journal of Pathology, 2010. **176**(3): p. 1241-1255.
31. Polyak, K. and R. Kalluri, *The Role of the Microenvironment in Mammary Gland Development and Cancer.* Cold Spring Harbor Perspectives in Biology, 2010. **2**(11): p. a003244.
32. Reed, J.R. and K.L. Schwertfeger, *Immune cell location and function during post-natal mammary gland development.* Journal of mammary gland biology and neoplasia, 2010. **15**(3): p. 329-339.
33. E., S., *The role of CSF-1 in normal physiology of mammary gland and breast cancer: an update. .* Experimental Biology and Medicine 2004. **229**: p. 1-11.
34. Lin, E.Y., Gouon-Evans, V., Nguyen, A. V. & Pollard, J. W., *The Macrophage Growth Factor CSF-1 in Mammary Gland Development and Tumor Progression.* Journal of Mammary Gland Biology and Neoplasia, 2002. **7**: p. 147-162.
35. Van Nguyen, A. and J.W. Pollard, *Colony Stimulating Factor-1 Is Required to Recruit Macrophages into the Mammary Gland to Facilitate Mammary Ductal Outgrowth.* Developmental Biology, 2002. **247**(1): p. 11-25.
36. MacLennan, M. and D.W.L. Ma, *Role of dietary fatty acids in mammary gland development and breast cancer.* Breast Cancer Research : BCR, 2010. **12**(5): p. 211-211.
37. Olson, L.K., et al., *Pubertal Exposure to High Fat Diet Causes Mouse Strain-dependent Alterations in Mammary Gland Development and Estrogen Responsiveness.* International journal of obesity (2005), 2010. **34**(9): p. 1415-1426.

38. Anderson, B.M., et al., *Lifelong exposure to n-3 PUFA affects pubertal mammary gland development.* Applied Physiology, Nutrition, and Metabolism, 2014. **39**(6): p. 699-706.
39. Hilakivi-Clarke, L., et al., *Mechanisms Mediating the Effects of Prepubertal (n-3) Polyunsaturated Fatty Acid Diet on Breast Cancer Risk in Rats.* The Journal of Nutrition, 2005. **135**(12): p. 2946S-2952S.
40. Ronnov-Jessen, L., O.W. Petersen, and M.J. Bissell, *Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction.* Physiological Reviews, 1996. **76**(1): p. 69.
41. Wang, A.T., et al., *Breast Density and Breast Cancer Risk: A Practical Review.* Mayo Clinic Proceedings. **89**(4): p. 548-557.
42. Russo, J.H., Y. F.; Yang, X.; Russo, I. H., *Developmental, cellular, and molecular basis of human breast cancer.* J. Natl. Cancer Inst. Monogr, 2000: p. 17-37.
43. Russell, T.D., et al., *Myoepithelial Cell Differentiation Markers in Ductal Carcinoma in Situ Progression.* The American Journal of Pathology, 2015. **185**(11): p. 3076-3089.
44. Carron EC, H.S., Rosenberg J, Coffelt SB, Kittrell F, Zhang Y, Creighton CJ, Fuqua SA, Medina D, Machado HL, *Macrophages promote the progression of premalignant mammary lesions to invasive cancer.* Oncotarget, 2017.
45. Mao, Y., et al., *Stroma Cells in Tumor Microenvironment and Breast Cancer.* Cancer metastasis reviews, 2013. **32**(0): p. 303-315.
46. Buchsbaum, R.J. and S.Y. Oh, *Breast Cancer-Associated Fibroblasts: Where We Are and Where We Need to Go.* Cancers, 2016. **8**(2): p. 19.
47. Gascard, P. and T.D. Tlsty, *Carcinoma-associated fibroblasts: orchestrating the composition of malignancy.* Genes & Development, 2016. **30**(9): p. 1002-1019.
48. Folgueira, Maria Aparecida A.K., et al., *Markers of breast cancer stromal fibroblasts in the primary tumour site associated with lymph node metastasis: a systematic review including our case series.* Bioscience Reports, 2013. **33**(6): p. e00085.
49. Biro, F.M. and J. Deardorff, *Identifying Opportunities for Cancer Prevention During Pre-Adolescence and Adolescence: Puberty as a Window of Susceptibility.* The Journal of adolescent health : official publication of the Society for Adolescent Medicine, 2013. **52**(5 0): p. S15-S20.
50. Colditz, G.A. and A.L. Frazier, *Models of breast cancer show that risk is set by events of early life: prevention efforts must shift focus.* Cancer Epidemiology Biomarkers & Prevention, 1995. **4**(5): p. 567.
51. Martinson, H.A., et al., *Developmental windows of breast cancer risk provide opportunities for targeted chemoprevention.* Experimental cell research, 2013. **319**(11): p. 1671-1678.
52. Sundaram, S., A.R. Johnson, and L. Makowski, *Obesity, metabolism and the microenvironment: Links to cancer.* Journal of Carcinogenesis, 2013. **12**: p. 19.
53. Dieli-Conwright, C.M., K. Lee, and J.L. Kiwata, *Reducing the Risk of Breast Cancer Recurrence: an Evaluation of the Effects and Mechanisms of Diet and Exercise.* Current Breast Cancer Reports, 2016. **8**(3): p. 139-150.
54. Shrivastava, S., P. Shrivastava, and J. Ramasamy, *Exploring the role of dietary factors in the development of breast cancer.* Journal of Cancer Research and Therapeutics, 2016. **12**(2): p. 493-497.
55. Yirong Zhu, M.D.A., Yong Zhao, Ying Siow Tan, Erin L. Kirk, and M.A.T. Xuezheng Sun, Richard C. Schwartz, Sandra Z. Haslam, *Pubertal and adult windows of susceptibility to a high animal fat diet in Trp53-null mammary tumorigenesis.* Oncotarget, 2016. **7**(50): p. 83409-83423.

56. Zhao, Y., et al., *Pubertal high fat diet: effects on mammary cancer development.* Breast Cancer Research : BCR, 2013. **15**(5): p. R100-R100.
57. Aupperlee, M.D., et al., *Puberty-specific promotion of mammary tumorigenesis by a high animal fat diet.* Breast Cancer Research : BCR, 2015. **17**: p. 138.
58. Jung, S., et al., *Dietary Fat Intake During Adolescence and Breast Density Among Young Women.* Cancer Epidemiology Biomarkers & Prevention, 2016. **25**(6): p. 918.
59. Paine, I., et al., *A Geometrically-Constrained Mathematical Model of Mammary Gland Ductal Elongation Reveals Novel Cellular Dynamics within the Terminal End Bud.* PLoS Computational Biology, 2016. **12**(4): p. e1004839.
60. McCuaig, R., et al., *The biological and clinical significance of stromal-epithelial interactions in breast cancer.* Pathology. **49**(2): p. 133-140.
61. Leena, H.-C., *Nutritional Modulation of Terminal End Buds: Its Relevance to Breast Cancer Prevention.* Current Cancer Drug Targets, 2007. **7**(5): p. 465-474.
62. Yang, L. and Y. Zhang, *Tumor-associated macrophages: from basic research to clinical application.* Journal of Hematology & Oncology, 2017. **10**: p. 58.
63. DeFilippis, R.A., et al., *Stress Signaling from Human Mammary Epithelial Cells Contributes to Phenotypes of Mammographic Density.* Cancer research, 2014. **74**(18): p. 5032-5044.